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# Combined Irradiation–Heat Processing of Canned Foods: Green Peas Inoculated with Anaerobic Bacterial Spores

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*Summary.* Combined irradiation–heat processing is shown to be synergistic for killing anaerobic bacterial spores inoculated into canned green peas. Following 1·2 megarad of gamma irradiation an $F_0$ of 0·5 sterilized green peas inoculated with either 5,000,000 *Clostridium botulinum* 213 B or 300 PA 3679 spores per can. This processing schedule must be considered a minimum since it is based on a limited number of cans. Because sterilization by either irradiation or heat alone can damage the organoleptic properties of canned foods, the lowered schedules possible with combined processing may prove to be of value for preserving vegetables as well as meats.

## Introduction

Combined irradiation–heat processing is shown to be synergistic for killing anaerobic bacterial spores inoculated into canned peas. This had previously been demonstrated with canned meat (Kempe *et al.*, 1957; 1959). It had also been shown that sterilizing doses of either heat or ionizing radiations often lower the organoleptic qualities of foods, but that smaller dosages may be acceptable (Schultz *et al.*, 1956). Since reduced quantities of gamma radiation and heat are required when their synergistic effect on bacterial spores is utilized, combined processing could become useful for food preservation. For this reason, it was considered desirable to learn whether combined irradiation–heat processing was also effective in canned vegetables. Green peas were selected for study because they are packed commercially and are a good growth medium for anaerobic bacterial spores (Reed *et al.*, 1951).



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## Materials and Methods

### *A. Packing*

Commercially frozen green peas were obtained in 2·5 lb boxes from the stock supply of the University of Michigan Food Service. Labels on the packages indicated that the peas contained a 'slight amount' of added salt.

In preparation for a run, 15 lb of the frozen peas were placed in a stock pot and these were covered with a brine containing 1·8 per cent sodium chloride and 2·2 per cent sucrose. This brine was previously described for peas by Reed *et al.* (1951). The stock pot was then placed in a boiling water bath for about 1·5 h during which time the frozen peas melted and were brought to a temperature of 205°F.

Number 1 picnic tin cans were filled with peas to within $\frac{1}{4}$ in. of the top; care was taken to cover the peas with brine. Covers were placed loosely on the cans that were then set in an autoclave which was filled with flowing steam. After the canned peas had been exhausted for a few minutes, individual cans were removed from the autoclave, inoculated at the geometrical centre with 1 ml of a spore suspension, sealed in a commercial-type closing machine, immersed in cold, running water for 20 min, and then refrigerated until they were either irradiated, heat-processed, or incubated at 85°F as required.

### *B. Irradiation*

The canned peas were irradiated in the centre-well of the large cobalt-60 gamma radiation source in the Fission Products Laboratory at the University of Michigan (Lewis *et al.*, 1954). The quantity of irradiation delivered at the centre of the cans was measured by ferrous–ferric sulphate dosimetry as previously described (Kempe *et al.*, 1954). At the time of this investigation, a dosage of 1 megarad* of gamma radiation required approximately 8 h exposure. When the temperature of the cave was above 40°F, the cans were refrigerated with dry ice to keep the peas below this temperature during irradiation. Following irradiation the cans were placed in a refrigerator at 35°F from which they were removed within 2 days for heat processing.

* One rad is a dose of ionizing radiation capable of producing energy absorption of 100 erg per g of tissue.

### *C. Heat Processing*

Six cans of meat, two of which contained thermocouples, were placed in an autoclave where they were heat processed to the desired $F_0$ values as previously described (Kempe *et al.*, 1957). The $F_0$ value is defined as the number of minutes required to sterilize the can of peas at 250°F when the $Z$ value equals 18. These values were calculated from time–temperature curves obtained from the cans containing thermocouples. For this purpose Schultz's graphical modification of Ball's General Method (Schultz and Olson, 1940) was used, and a $Z$ value of 18 was assumed. Following heat processing, the cans were incubated at 85°F.

### *D. Spores*

The spores of anaerobic bacteria used in these studies were prepared and used as previously described (Kempe *et al.*, 1954).

### *E. Controls*

Eight control cans were used. Four of these were not inoculated and four were selected at random from the experimental cans. All eight were then incubated at 85°F. Generally the non-inoculated cans swelled within three weeks while gas usually developed within one week in the inoculated cans. This indicated that conditions suitable for microbiological growth were present. However, this did not conclusively demonstrate viability of the inoculum in the peas, since the non-inoculated peas contained bacteria.

When *Clostridium botulinum* 213 B was used for the inoculum, mouse inoculation tests established the presence or absence of B type toxin in the peas (Kempe *et al.*, 1954). Occasionally this type of toxin was also recovered from non-inoculated control cans which indicated that the peas contained *C. botulinum* type B spores when originally frozen. For this reason, the $F_0$ required to sterilize non-inoculated canned peas was established by control runs.

## Results

Data for a typical run are shown in Table I. Two series of runs were conducted. In the first series, shown in Table II and Fig. 1,

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Table I. $F_0$ values required to sterilize canned green peas packed in No. 1 picnic tin cans, inoculated with PA 3679 spores, and irradiated with gamma rays from cobalt-60 before heat processing

| | |
|---|---|
| Run No. CP 6 | |
| Can Size | No. 1 Picnic (211 × 400) |
| Product | Green Peas |
| Inoculum | 300 PA 3679 spores per can |
| Irradiation | 0·279 megarad |
| Processing temperature | 230°F |
| Incubation temperature | 85°F |

| $F_0$ | Can No. | Days to gas formation |
|---|---|---|
| 0·77 | 13 | 4 |
| 0·77 | 14 | 4 |
| 0·77 | 15 | 4 |
| 0·77 | 16 | 4 |
| 1·47 | 17 | 5 |
| 1·47 | 18 | 4 |
| 1·47 | 19 | 4 |
| 1·47 | 20 | 5 |
| 2·19 | 21 | — |
| 2·19 | 22 | 5 |
| 2·19 | 23 | — |
| 2·19 | 24 | — |
| 3·16 | 9 | — |
| 3·16 | 10 | — |
| 3·16 | 11 | — |
| 3·16 | 12 | — |
| Controls: | 1 | 3 |
| Not inoculated | 2 | — |
| | 3 | 23 |
| | 4 | 6 |
| Controls: | | |
| Inoculated | 5 | 3 |
| | 6 | 3 |
| | 7 | 6 |
| | 8 | 7 |

Conclusions: Following 0·279 megarad of gamma radiation from cobalt-60, canned peas were sterilized by an $F_0$ between 2·2 and 3·2.

Table II. Combined irradiation–heat processing treatments required to sterilize canned green peas packed in No. 1 picnic tin cans and inoculated with anaerobic bacterial spores

| Run No. | Pre-irradiation megarad | $F_0$ range min |
|---|---|---|
| a) Series 1: 5,000,000 *C. botulinum* 213 B spores per can | | |
| PB 6 | 2.33–2.79 | None |
| PB10 | > 2.79 | None |
| PB19 | 2.79–3.29 | None |
| PB11 | None | < 1.54 |
| PB 4 | None | 1·39–1·82 |
| PB 1 | None | > 1·44 |
| PB 3 | 0·465 | 1·03–1·53 |
| PB14 | 0·465 | 0·60–0·75 |
| PB15 | 0·650 | 0·80–0·92 |
| PB 5 | 0·930 | 0·36–0·50 |
| PB 8 | 1·40 | 0·33–0·48 |
| PB18 | 1·63 | < 0·30 |
| PB17 | 1·96 | < 0·06 |
| b) Series 2: 300 PA 3679 spores per can | | |
| CP 2 | 1·77–2·05 | None |
| CP 1 | None | 4·1–4·9 |
| CP 6 | 0·279 | 2·2–3·2 |
| CP 3 | 0·465 | 1·39–2·25 |
| CP 5 | 0·697 | 0·33–0·69 |
| CP 4 | 0·930 | 0·25–0·49 |
| Non inoculated controls | | |
| PB 7 | None | > 0·06 |
| PB 9 | None | > 0·054 |
| PB12 | None | 0·35–0·52 |
| PB13 | None | > 0·20 |
| PB15 | None | 0·10–1·10 |
| PB 7 | > 0·465 | None |
| PB15 | > 0·650 | None |

5,000,000 *C. botulinum* 213 B spores were used per No. 1 can of peas. In the second series, shown in Table II and Fig. 2, 300 PA 3679 spores were similarly used. These data show that gamma radiation and heat are synergistic when used together for processing canned peas. Less of either form of energy was needed for sterilization when combined with the other than when used alone.

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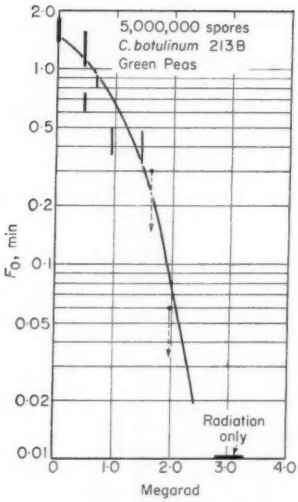


Fig. 1. $F_0$ required to sterilize green peas packed in No. 1 picnic tin cans, inoculated with 5,000,000 *C. botulinum* 213 B spores per can and irradiated with gamma rays from cobalt-60 before heat processing

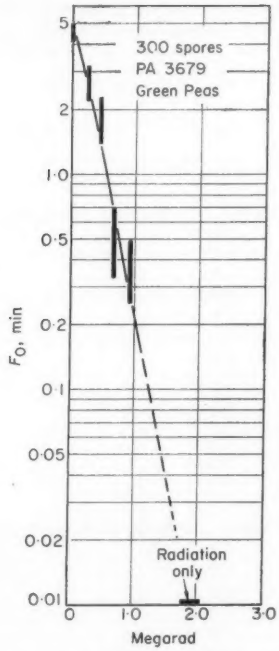


Fig. 2. $F_0$ required to sterilize green peas packed in No. 1 picnic tin cans, inoculated with 300 PA 3679 spores per can, and irradiated with gamma rays from cobalt-60 before heat processing

Following 1·2 megarad of gamma radiation an $F_0$ of 0·5 sterilized the peas whether they were inoculated with 5,000,000 *C. botulinum* 213 B or 300 PA 3679 spores. Below 1 megarad pre-irradiation, more heat processing was required to sterilize cans of peas inoculated with 300 PA 3679 spores than was necessary when 5,000,000 *C. botulinum* 213 B spores were used; above 1 megarad pre-irradiation, the reverse was true.

## Discussion

The synergistic lethal action of gamma radiation is probably a

general phenomenon since it is shown to be essentially as pronounced for spores suspended in canned green peas as it has previously been reported to be for such spores suspended in canned beef and in phosphate buffer (Kempe *et al.*, 1957; 1955). It would therefore appear reasonable from a microbiological viewpoint that this synergistic lethal property could be expected to be applied to the sterilization of most canned foods. It also appears that the synergistic action results from sensitization of the spores to heat as a result of irradiation. This effect varies in degree according to the medium in which the spores are suspended so wherever utilization of the effect is considered, the actual application should be studied.

It must be pointed out that the combined irradiation–heat processing treatments reported here are minimum values because they are based on a limited number of runs and upon four cans at each level as shown in Table I.

Relevant to the possible improvement in organoleptic values to be derived from combined irradiation–heat processing, Gillies (1959) has recently reported on studies with canned peas. He found that thermally processed peas were superior to those processed either by gamma radiation alone or by combined irradiation–heat processing. However, he also stated that combination processed peas were significantly better, in most cases, than those processed with radiation alone.

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# Glycerol Production: A Pilot-Plant Investigation for Continuous Fermentation and Recovery*†

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*Summary.* A pilot plant was built to evaluate the economic potentialities of producing glycerol from sugar by the bisulphite fermentation process. The design of this small unit was based on information collected from bench-scale studies of the fermentation and recovery processes taking advantage of new separation techniques.

A beer containing 5 per cent of glycerol is produced in a continuous fermentation in which the free bisulphite level is automatically controlled. The effluent from the fermentor is centrifuged, and the yeast is returned to the fermentation. The dilute clarified beer is acidified; sulphur dioxide, acetaldehyde, and alcohol are removed; and the stripped solution is neutralized and concentrated until it contains 45 per cent of glycerol. This concentrated glycerol stock is purified by using a combination of ion-exclusion and ion-exchange.

Good fermentation control results in a beer that has a low solids-to-glycerol ratio, low organic acid content, and low residual sugar. The method of glycerol purification results in a high glycerol recovery, since no distillation of glycerol is involved. The sulphur dioxide removed from the beer is recycled through the fermentation so that the chief raw materials used in the process are sugar, sulphuric acid, and sodium carbonate. The yields of products based on sugar charged are: glycerol, 25 per cent; alcohol, 17 per cent; and acetaldehyde, 11 per cent.

The economic success of the process depends to a large extent upon the price of sugar. Under present conditions it is felt that the process is competitive.

## Introduction

The studies directed at elucidating the mechanism of the yeast fermentation, which were carried out by Neuberg and co-workers [5,6]

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around 1911, revealed the possibility of producing glycerol by the addition of sulphite salts to the normal alcohol fermentation. Acetaldehyde, formed as an intermediate during the normal course of the fermentation, reacts with bisulphite salt and is effectively removed from the fermentation. The enzyme system that would normally reduce acetaldehyde to alcohol now reduces another intermediate, dihydroxyacetone, to glycerol, and the end-products are glycerol, acetaldehyde, and carbon dioxide.

Shortly after this basic discovery of Neuberg's, the First World War began and the glycerol supply became crucial. During hostilities, investigations were started on both sides of the Atlantic[2,3] to develop a production method based on Neuberg's findings. In Germany a process was hastily developed to the point of commercial production, with output running above 1,000 tons of glycerol per month. The process was poorly designed, however, and did not survive subsequent peace-time competition. During the period between World War I and World War II, there was little interest in the production of glycerol by such processes, but with the arrival of the Second World War the urgency of the glycerol demand was again acute. Research in England, the United States, and Canada was revived, but during the hostilities no processes of commercial promise resulted.

The capture of the detergent market by syndets, following World War II, threatened to decrease the glycerol supply below normal peace-time requirements. The research in Britain on the production of glycerol by fermentation resulted in the development of an industrial scale process that is now available for licensing. In the United States a synthetic process based on propylene was developed and a plant built in 1948. At that time, it became apparent that the future glycerol demand would be supplied from relatively few, perhaps even one, synthetic plants with large capacity. The military services were interested in developing an alternative process that would produce glycerol at reasonable costs, in widely dispersed plants, from easily available, domestic, non-critical raw materials. The U.S. Army Ordnance therefore entered into contract research with the Forest Products Laboratory to develop a suitable process or processes. This paper reports on the portion of that work related to the production of glycerol by the sodium bisulphite 'steered' fermentation process.

### Sodium Bisulphite–Acetaldehyde Reaction

The reaction between sodium bisulphite and acetaldehyde is of such basic importance to the process that a brief discussion of the equilibrium is necessary. It must be understood in order to interpret the results of changing process variables in the fermentation, in the removal of volatile constituents from the fermented beer, and in the recovery of acetaldehyde and sulphur dioxide.

The reaction is illustrated in Fig. 1, which shows the generally accepted formulae for the compounds involved. There has been considerable discussion about the exact structure of aldehyde–bisulphite complexes, but it has been proved[8,9] that they are α-hydroxysulphonic acids, as illustrated. Thus, they exhibit a high degree of ionization, possessing an acid strength equivalent to that of hydrochloric acid. Consequently, the concentration of the undissociated complex in aqueous solutions is always small relative to the concentration of the complex ion. This is shown by the second equation of Fig. 1.

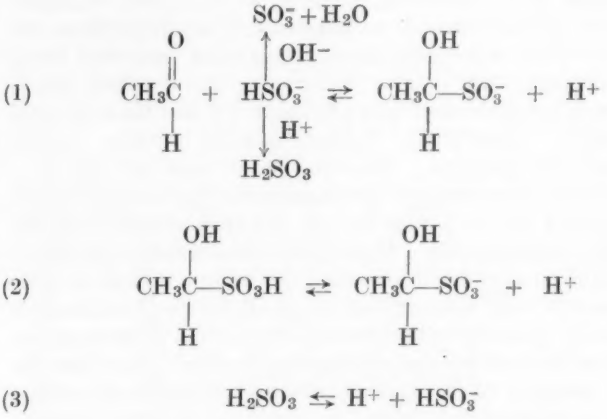



Fig. 1. The acetaldehyde–bisulphite equilibrium.

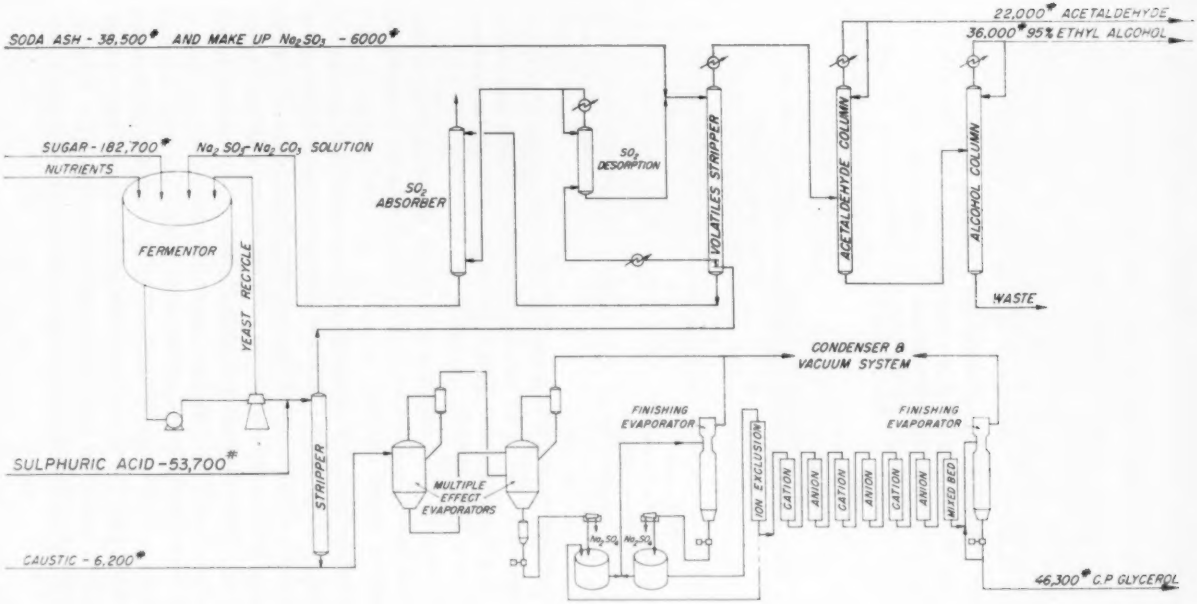
From equation (1) it is evident that the quantity of the complex ion in solution is dependent on the concentration of bisulphite ion. As this concentration is dependent on the solution pH, the

proportions of 'free' and 'bound' sulphur also depend on the pH. The addition of acid to a solution of the complex causes a shift to the left in the equilibrium, shown in equation (1), with an increase in the concentration of sulphurous acid. Thus, in highly acidified solutions the acetaldehyde and sulphur dioxide exert high vapour pressures, approaching that which they would exert if no complexing occurred. The addition of base also decreases the concentration of bisulphite ion, shifting the equilibrium to the left. In this case, the sulphur is present largely as the sulphite ion, and the partial pressure of sulphur dioxide above the solution is quite small. The acetaldehyde is uncombined and exhibits a high partial pressure.

## Process Description

The process flow sheet is shown in Fig. 2. Sugar solution is continuously charged to the fermentors, together with the necessary nutrients, yeast, sodium carbonate, and sodium bisulphite. The resultant beer, containing glycerol, alcohol, and combined acetaldehyde, is continuously withdrawn and separated from the yeast. This yeast is returned to the fermenting vats after being given a mild acid wash. After centrifuging, the clarified beer is acidified with sulphuric acid to a pH below 1·0 and then stripped of its volatile constituents, alcohol, sulphur dioxide, carbon dioxide, and acetaldehyde. The volatiles are sent to a recovery unit where they are separated by a sequence of fractionation steps, employing soda ash, to adjust the pH and thus separate sulphur dioxide from acetaldehyde. This will be discussed in more detail, but it should be mentioned here that the sodium added to effect this recovery is only that amount required for the fermentation and is equal in quantity to that leaving the bottom of the stripper.

The dilute glycerol stream, approximately 5 per cent, is neutralized and charged to a multiple effect evaporator–crystallizer where its concentration is raised to about 60 per cent glycerol. The economics of the recovery depend to a large extent on the performance of this evaporator. It must produce a product stream with a high glycerol-to-impurity ratio for subsequent processing. This is complicated by the high viscosity of the solution, arising from the presence of residual sugar, sodium lactate, and




FLOW SHEET FOR 15,000,000# PER YEAR FERMENTATION GLYCEROL PLANT

Fig. 2. Flow sheet of the fermentation glycerol process. # = lb or other convenient units.

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sodium acetate. These minor constituents of the fermentation are of much importance here, emphasizing the necessity of maintaining good fermentation control. During the evaporation, sodium sulphate is removed from the solution as the decahydrate, which is converted in another evaporator to the anhydrous salt.

Table I. Fermentation glycerol process

| Component | lb |
|---|---|
| Raw Materials | |
| Sugar | 3,910 |
| Sulphuric acid | 1,070 |
| Soda ash | 1,055 |
| Sodium sulphite (make-up) | 105 |
| Ion exchange regenerants: | |
| Sulphuric acid | 38 |
| Sodium hydroxide | 106 |
| Products | |
| Glycerol | 1,000 |
| Ethyl alcohol | 650 |
| Acetaldehyde | 420 |
| Sodium sulphate (anhydrous) | 1,150 |

The evaporator product is processed through an ion-exclusion, ion-exchange unit that removes the remainder of the impurities accompanying the glycerol. The aqueous glycerol solution is then evaporated to a high-quality product.

The raw material requirements and the products of the process are shown in Table I. The overall yields of organic compounds on the basis of sugar charged are: 25·6 per cent glycerol, 16·6 per cent ethyl alcohol, and 10·7 per cent acetaldehyde, a total of 52·9 per cent. These figures are based on a 90 per cent recovery of glycerol and 80 per cent recovery of ethyl alcohol and acetaldehyde from the fermentation beer. In addition to these organic products, anhydrous sodium sulphate of high purity is produced in the amount of 0·294 lb per lb of sugar charged.

The raw materials required, other than sugar, are soda ash and sulphuric acid. Make-up sodium sulphite is also required to compensate for the sulphur dioxide not removed in the primary stripping operation. As well as the raw materials shown in Table I, minor quantities of nutrients must be added to the fermentation.

### *Fermentation*

A review of the development of fermentation procedures for glycerol production has been given by Underkofler.[11] The fermentation method employed here is fundamentally similar to the various bisulphite-addition fermentations described by him, but with several important modifications. The process was put on a continuous basis, and equipment was supplied to automatically control pH, bisulphite ion concentration, and temperature. The pertinent information relative to the fermentation is given in Table II.

Table II. Glycerol fermentation

Organism: *Saccharomyces cerevisiae* (FPL No. 49)
Temperature: 32°C
pH: 6·5

| Input | lb | Output | lb |
|---|---|---|---|
| Sugar (20% solution) | 3,521 | Glycerol | 1,000 |
| Yeast (80% moisture) | 390 | Acetaldehyde | 473 |
| Nutrients: | | Ethyl alcohol | 735 |
| urea | 14·6 | Sodium bisulphite: | |
| disodium phosphate | 9·4 | bound | 1,117 |
| potassium chloride | 3·1 | free | 13 |
| magnesium sulphate | 0·8 | Residual sugar | 32 |
| blackstrap molasses | 48·0 | Organic acids | 36 |
| Sodium bisulphite | 1,130 | Inorganic salts | 21 |
| Sodium carbonate | 281 | | |

Underkofler[11] repeatedly points out that the main problem in commercial development of the fermentation production of glycerol is the difficulty in separating the product from large amounts of salts and other materials present in the fermented beer. This is an important point. In Table II it is seen that the glycerol is accompanied by alcohol, acetaldehyde, residual sugar, sodium lactate, and sodium acetate. Careful analysis for these components, together with the carbon dioxide produced in the fermentor, have accounted for 99 per cent of the carbon in the sugar charged. Thus, these compounds were assumed to be the only components present, except for small amounts of protein coming

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into solution from the yeast. This assumption was further substantiated by calculating the oxidation–reduction balance that gave an O/R ratio of 0·993.

The yield of glycerol on sugar charged is 55·51 per cent of that theoretically attainable and that of alcohol, 40·83 per cent of the theoretical. The residual 3·76 per cent is accounted for in the residual sugar and organic acids. Acetaldehyde is produced in almost a mole per mole ratio with the glycerol, its yield being 55·16 per cent of theoretical. These yields of products change only slightly over rather large variations in fermentation conditions. Thus, control of the fermentation is comparatively simple. Variations in the controlled variables have a much larger effect proportionately on the amount of minor organic constituents present than on the yields of primary products.

The sodium acetate present in the broth results from small amounts of acetaldehyde undergoing the Cannizzaro reaction, as suggested by Neuberg and co-workers. This reaction becomes much more prominent if the pH of the fermenting medium is raised, and will be insignificant if the pH is decreased. However, a decrease in the pH results in a pronounced decrease in the rate of fermentation.

Sodium bisulphite and soda ash are added to the fermentation to control the pH and bisulphite ion concentration of the solution. There is, of course, interaction between these variables. It is important to maintain close control over the bisulphite ion concentration for several reasons. Low concentrations of bisulphite ion are ineffective in binding the acetaldehyde; consequently, the yields of glycerol decrease and alcohol yields increase. High bisulphite levels decrease the activity of the yeast, resulting in slower rates. Also the residual sugar level increases. This results in lower yields on charged sugar and increased difficulty in recovering the glycerol. Control of the bisulphite ion level is accomplished by continuous withdrawal and titration of a small sample stream from the fermentor.

The presence of sodium lactate in the fermentor product indicates the presence of bacterial contamination. These bacteria are not active in the fermentor, for the presence of the sulphite maintains aseptic conditions. They are active in the recycle yeast equipment where the bisulphite ion is not present in sufficient

quantity to retard their activity. The yeast is centrifuged from the beer and returned to the fermentors; there is a small increase in yeast volume for each cycle. Before being returned, it is acid washed[1] and rejuvenated. To be rejuvenated, it is mixed with the incoming sugar feed to the fermentor and allowed approximately half an hour to become active. It is then added with the sugar to the fermentors where it again comes in contact with bisulphite ion. If bacteria are present during this activating time, they will produce some lactic acid but will become dormant as soon as they are introduced to the main medium. Proper handling of the yeast will eliminate lactates entirely. In the experimental work, yeast was recycled daily for a year with no change in strain or accumulation of contaminating organisms. There is a small increase in yeast in each cycle.

The amount of residual sugar is brought to a minimum value by aeration, in a second vat, following the main fermentation. The holding time in the main fermentor is 24 h, and at this throughput rate the sugar concentration in the effluent is approximately 1 per cent. The solution is then held in the second fermentor for an additional period of 12 h. The introduction of a small amount of air during this period reduces the residual concentration to less than 0·10 per cent.

*Removal and Recovery of Volatiles*

The clarified solution from the fermentation is acidified with strong sulphuric acid and charged to a stripping column. The quantity of acid added must be sufficient to reduce the pH to less than 1·0. As previously explained, the complex is highly dissociated at this pH level, and the sulphur dioxide and acetaldehyde exert nearly normal vapour pressures. The amount of acid added amounts to approximately 1·07 lb of sulphuric acid per lb of glycerol.

During the stripping operation, ethyl alcohol, acetaldehyde, sulphur dioxide, and carbon dioxide are all removed in the overhead. The energy requirement for this operation is approximately 3·5 lb of steam per lb of glycerol. The dissociation rate of the complex is high and is not a limiting factor in the stripper operation. The association of sulphur dioxide and acetaldehyde lowers the effective vapour pressure of these components, which

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necessarily increases column size above that required if no association occurred. Using the conditions described, 95 per cent removal of all volatile components can be easily accomplished.

The corrosive nature of this solution after acidification is quite apparent. Construction of the tower and packing must necessarily be of a corrosion-resistant material. Immediately following removal of volatiles, the solution is neutralized. If the neutralizing stream is introduced into the still portion of the stripping column, the corrosive conditions are limited to the tower and packing.

Table III. Primary stripping column

| Component | Composition by weight | | |
|---|---|---|---|
| | Feed % | Overhead % | Bottoms % |
| Ethyl alcohol | 3·33 | 17·63 | 0·08 |
| Acetaldehyde | 2·15 | 11·36 | 0·05 |
| Sulphur dioxide | 3·15 | 16·69 | 0·08 |
| Carbon dioxide | 0·51 | 2·86 | – |
| Acetic acid | 0·09 | 0·05 | 0·08 |
| Water | 79·68 | 51·41 | 86·09 |
| Sodium sulphate | 5·21 | – | 6·39 |
| Residual sugar | 0·14 | – | 0·18 |
| Sulphuric acid | 0·76 | – | 0·95 |
| Protein | 0·10 | – | 0·12 |
| Lactic acid | 0·10 | – | 0·12 |
| Other organics | 0·26 | – | 0·32 |
| Glycerol | 4·52 | – | 5·56 |

The composition of the streams associated with the stripper is shown in Table III. The overhead stream of the composition shown is processed, using ordinary distillation equipment, to recover the components: sulphur dioxide, acetaldehyde, and ethyl alcohol. The first step in the separation is partial condensation with the separation of a portion, approximately 25 per cent, of the incoming sulphur dioxide, as a pure aqueous solution. Separation of the acetaldehyde and alcohol from the remaining sulphur

dioxide is then accomplished by adding soda ash. This effectively binds the sulphur dioxide, and the alcohol and acetaldehyde are removed and rectified further to obtain the pure components. The alkaline stream is then used to absorb the sulphur dioxide removed previously.

The initial removal of sulphur dioxide by fractional condensation is necessary to maintain the sodium–to–sulphur dioxide ratio at the same value as that required in the fermentation while maintaining sodium addition at a minimum value. If the entire sulphur dioxide was kept in the solution and the solution brought to pH 9·0, the alkalinity of the recycle stream to the fermentors would be much too high. This would require the addition of sodium bisulphite, resulting in a net increase in the amount of recycle. This can be avoided by the partial removal of sulphur dioxide prior to acetaldehyde removal.

*Concentration of Sodium Sulphate: Glycerol Solutions*

The neutral solution from the stripper is evaporated to a 60 per cent glycerol concentrate. This operation can be conveniently considered in four distinct parts: concentration to the saturation point, crystallizing the sodium sulphate decahydrate, finishing to a high glycerol concentration, and conversion of the decahydrate to the anhydrous salt.

In the first step, the concentration to the saturation point, the solution is concentrated to about 13 per cent glycerol. As this requires evaporating 65 per cent of the water in the feed, the major heat load occurs in this first step. This job can be accomplished using conventional evaporators of steel construction. The small amounts of contaminants that may result from minor corrosion are unimportant in subsequent processing. The physical properties of the solution are approximately the same as those of glycerol solutions encountered in the by-product soap glycerine industry.[4]

In the crystallizing evaporators the glycerol concentration is maintained at 30 per cent. At this concentration the sodium sulphate precipitates as the decahydrate. At higher concentrations it precipitates as small crystals of the anhydrous salt, which are difficult to wash free of the viscous glycerol solution.

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For this reason the solids precipitated in the finishing evaporators are recycled back to the crystallizer, and all the sodium sulphate removed during the evaporation is removed as the decahydrate. This salt, after washing, can be handled in the usual manner to produce the anhydrous salt.

The compositions of the various streams involved in the evaporation step are presented in Table IV.

Table IV. Evaporator–crystallizer

| Component | % Composition by weight | | | |
|---|---|---|---|---|
| | Evaporator feed | Crystallizer feed | Finisher feed | Finisher product |
| Ethyl alcohol | 0·08 | – | – | – |
| Acetaldehyde | 0·05 | 0·12 | 0·29 | 0·57 |
| Sodium sulphite | 0·15 | 0·36 | 0·84 | 1·65 |
| Sodium sulphate | 7·72 | 18·15 | 17·62 | 14·54 |
| Sodium lactate | 0·12 | 0·27 | 0·64 | 1·26 |
| Sodium acetate | 0·08 | 0·19 | 0·46 | 0·90 |
| Residual sugar | 0·18 | 0·41 | 0·97 | 1·89 |
| Protein | 0·12 | 0·27 | 0·64 | 1·26 |
| Other organics | 0·31 | 0·74 | 1·74 | 3·41 |
| Water | 85·66 | 66·49 | 46·20 | 14·52 |
| Glycerol | 5·53 | 13·00 | 30·60 | 60·00 |

*Recovery of Glycerol from Concentrated Solutions*

The recovery procedures suggested by the patent literature are nearly all based on solvent extractions or distillation. Early investigations proved that each of these methods has serious disadvantages when used with this feed stock. In the case of solvent extraction, suitable solvents with high selectivity and high glycerol uptake could not be found. If distillation is used, the high viscosity of the bottoms necessitates designing special equipment. Losses in the still bottoms and the decrease in product quality are important considerations. The particular method chosen for the pilot unit at this Laboratory was a combination of ion-exclusion, ion-exchange, and evaporation—similar to that described by Prielipp and Keller.[7] This recovery scheme has the advantages of high yield and high product quality. It has the

further advantage that it is capable of handling large variations in feed stock.

The ion-exclusion technique is basically a selective absorption or chromatographic separation; non-ionic components are strongly absorbed by ion-exchange materials, whereas ionic components are almost totally excluded from the resin phase. The method differs from the usual ion-exchange, as no chemical reaction takes

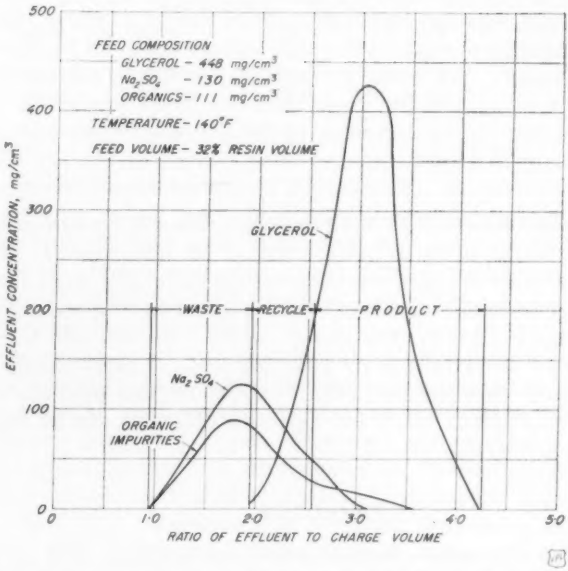


Fig. 3. Concentration–effluent volume relations for a typical ion-exclusion cycle.

place. The process consists of alternately feeding the solution, which contains the solutes to be separated, and pure solvent, to a column of ion-exchange material. As the solution proceeds down the column, a separation occurs because the non-ionic components will be absorbed on the resin and be retarded.

Typical effluent concentration curves for this stock are shown in Fig. 3. The separation of the glycerol from salt and also extraneous organic impurities is apparent. Separation of these organic impurities indicates that they are either ionic impurities,

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such as lactates or acetates, or have too large a molecular structure to enter the pore structure of the resin. The compositions of the various streams are shown in Table V.

Table V. Ion-exclusion

| Component | % Composition by weight | | | |
|---|---|---|---|---|
| | Feed | Waste | Recycle | Product |
| Ionic impurities | 10·84 | 7·22 | 4·84 | 0·515 |
| Non-ionic impurities | 9·02 | 6·65 | 3·17 | 0·702 |
| Glycerol | 37·40 | 0·90 | 4·97 | 21·30 |
| Water | 42·74 | 85·23 | 87·02 | 87·48 |
| Volume ratio | 1·0 | 1·0 | 0·65 | 1·6 |

The product stream from the exclusion column is ion-exchanged to obtain an aqueous glycerol solution of high purity. The exchange unit consisted of two cation-anion pairs and a mixed bed.

In the pilot-plant work the product obtained on evaporating the ion-exchanged solution was water white. It met all U.S.P. specifications except the silver reducing test. Unfortunately, it was necessary to discontinue the work before this problem was investigated further. It is felt that this difficulty can be easily overcome.

## Project State

From the viewpoint of commercial development, each of the process steps described above are in different stages of development. In general, those near the beginning are more advanced than those in the later part of the process. An idea of the overall state of development can be obtained by listing the studies that might be done before the process could be considered ready for commercial production.

A purely academic study of the acetaldehyde–bisulphite complex would be desirable. Although there is considerable work reported in the literature concerning the structure of this compound[8,9] no explanation has been given for its very high stability. Measurements on the dissociation constants have been

reported[10] but measurements on the rates of dissociation are not available. Reliable data of this type could be used to design the primary stripper and the recovery unit.

Before settling on an industrial design, the ion-exclusion, ion-exchange recovery unit described should be compared with other schemes for separating glycerol from the evaporated stock. Recently several new stills suitable for glycerol distillation have appeared on the market. A combination of ion-exclusion and distillation might prove to be less costly. It is also possible that cation exchange of the concentrate, followed by lime precipitation of the sulphate ion would be a satisfactory procedure. Regeneration of the exchanger could be done using sulphurous acid from the volatile recovery unit, thereby reducing the overall cost. The feasibility of this method could be inferred from data collected on the acetaldehyde–bisulphite equilibrium. It has already been noted that using the ion-exclusion, ion-exchange method, as described, results in a product which does not meet U.S.P. specifications. If this procedure is used, further experimental work is needed to determine how the final product can be upgraded to meet these specifications.

Throughout most of the work in the pilot unit in Madison, corn sugar was used as the charge material to the fermentor. All of the experimental work on the recovery unit was done with stocks generated from corn sugar. However, the possibility of using blackstrap molasses was investigated in the fermentation step. When molasses were used to replace the corn sugar the consumption of sodium bisulphite was increased, but this was the only effect. The additional consumption of sodium bisulphite was accounted for by the precipitation of calcium sulphite which built up in the recycling yeast stream. It is felt that the best way of overcoming this difficulty is to remove the calcium before charging the molasses to the fermentor.

## Process Potential

The technical status of the process outlined is good. That is, no insurmountable engineering or technical problems became apparent in any of the work. In fact, the process steps appear rather straightforward.

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A careful preliminary economic evaluation of the process (based on a 15,000,000 lb per year plant) indicates that glycerol could be produced well below the current market price, at the present price of hi-test molasses. A direct comparison of this process with the synthetic processes indicate that at the current prices for propylene and hi-test molasses, this competition could be met successfully. With the possibility of developing this process to utilize blackstrap molasses, it is felt that the process deserves commercial consideration.

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# An Attempt to Determine Discriminate Use of Hydrogen Isotopes by Bacteria

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*Summary.* The hydrogenase system of *Hydrogenomonas facilis* could be induced by either hydrogen or deuterium and the rate of deuterium uptake by the induced cells was the same as that of hydrogen.

An attempt has been made to detect discriminate use of hydrogen and deuterium by bacteria. No discrimination could be detected during hydrogenation of methylene blue by *Escherichia coli*, under the experimental conditions.

## Introduction

Non-biological hydrogenation reactions often show an isotope effect; in some, hydrogen reacts 2–3 times as quickly as deuterium (Wiberg 1955). Some bacteria catalyse hydrogenation reactions and it was of interest to see whether these, too, showed an isotope effect. In this work we used *Escherichia coli* and *Hydrogenomonas facilis*. These bacteria also catalyse an exchange reaction between gaseous hydrogen or deuterium and the hydrogen or deuterium in water molecules (Farkas, Farkas and Yudkin, 1934) and it was necessary to distinguish between this reaction and the hydrogenation reactions. The hydrogenase system of *Hydrogenomonas* is induced in the presence of hydrogen and it was possible to compare the behaviour of cells induced with hydrogen with that of cells induced in the presence of deuterium.

## Method

### *Preparation of Deuterium, and Deuterium–Hydrogen Gas Mixtures*

Deuterium was prepared by electrolysis of heavy water (containing 99·8 per cent $D_2O$) which had been rendered alkaline by

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the addition of metallic sodium. The deuterium was drawn from the electrolytic cell into a glass vessel which had previously been evacuated, and then partially filled with hydrogen to a known pressure. After the deuterium had all passed into this mixing vessel and its pressure had been measured, hydrogen was again added until the contents were at atmospheric pressure. An 18 V DC current was then passed through the gas mixture for 20 min to bring the gas into isotopic equilibrium. A current was also passed through the gas samples collected during the experiments to ensure equilibrium before analysis. The concentration of deuterium in the gas samples was determined by mass spectrometry. We are grateful to Dr. Bunton of the Department of Chemistry, University College, London, for carrying out these analyses.

*Experiments with* Hydrogenomonas facilis

The cells were grown at 25°C on heterotrophic agar (Schatz and Bovell, 1952) and harvested after 45 h. They were suspended in 0·033 M Sorensen phosphate buffer, pH 7·2. These cells contained no appreciable hydrogenase activity, but this could be induced by shaking the cells in an atmosphere of 95 per cent $H_2$/5 per cent air (v/v) for 24 h (Linday and Syrett, 1958). Hydrogenase activity was also induced by shaking in 95 per cent $D_2$/5 per cent air (v/v) and the activity of cells induced in this way was compared with that of cells induced in the hydrogen/air mixture. The results are shown in Table I. There is a suggestion in experiments 1 and 2, that less hydrogenase activity was induced by deuterium compared with hydrogen. Nevertheless, this is not borne out by experiment 3 and in fact, statistical analysis shows that the difference, taking the data as a whole, is not significant. However the cells are induced, the rates at which they take up hydrogen or deuterium are clearly the same.

*Experiments with* E. coli (*Strain 86*)

The cells were grown at 37°C, in Lemco peptone broth containing 1 per cent glucose. After 16 h, they were harvested by centrifugation, washed and suspended in 0·2 M phosphate buffer, pH 7·2, by passage of a stream of hydrogen. The experiments were carried out at 37°C, in Warburg flasks of 200 ml capacity

Table I. The induction of hydrogenase activity in *H. facilis* by incubation with either hydrogen or deuterium

| Incubation gas mixture | Gas during expt. | Rates of gas uptake | | |
|---|---|---|---|---|
| | | expt. 1 | expt. 2 | expt. 3 |
| 95% $H_2$+5% Air | $H_2$ | 148 | 115 | 135 |
| | $D_2$ | 134 | 110 | 130 |
| 95% $D_2$+5% Air | $H_2$ | 125 | 80 | 140 |
| | $D_2$ | 142 | 75 | 140 |

*H. facilis*, suspended in 0·033 M phosphate, pH 7·2, was incubated for 24 h at 25°C under a mixture of 95 per cent $H_2$/5 per cent air (v/v) or 95 per cent $D_2$/5 per cent air (v/v.)
Hydrogenase activity was measured on 2 ml samples of the cell suspension after incubation, in an atmosphere of either 100 per cent $H_2$ or 100 per cent $D_2$ with 0·008 M methylene blue as acceptor. The centre wells of the Warburg flasks contained 0·1 ml alkaline pyrogallol to remove traces of oxygen.
The rate of gas uptake is expressed as ml/h/mg dry wt.

attached to conventional manometers containing mercury as manometer fluid. Each flask contained 4 mg dry weight of cells. Methylene blue was added to the flasks in which hydrogenation was to be measured so that its final concentration was 0·033 M; preliminary experiments showed that higher concentrations of methylene blue were inhibitory. Methylene blue was omitted if the exchange reaction only was to be measured. The flasks were filled with hydrogen containing about 1 per cent atom excess deuterium by an evacuation method and during the reaction, gas samples of about 5 ml were removed through the side-arm stopper into evacuated sample bulbs for subsequent analysis. Under these conditions, the rate of hydrogen uptake was about 4·0 ml/h/flask.

The analysis showed no change in the percentage of deuterium in the gas phase over an experimental period of 90 min; for example, in one experiment the initial ratio of D/H in the gas was 0·620, after 30 min it was 0·618, after 60, 0·630, at the end of the experiment 0·620. Longer experiments were not possible since, by this time, all the methylene blue had been reduced. However, from this data, it is not possible to conclude with certainty that no discrimination between hydrogen and deuterium occurred since the total volume of hydrogen taken up was only 3 per cent of the volume in the flask.

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Some measurements were also made of the catalysis of the exchange reaction between deuterium in water and hydrogen of the gas phase. These measurements were made using the large Warburg flasks. $D_2O$ was added to the bacterial suspension so that its final concentration was 1·0 per cent (v/v). The gas phase was initially 100 per cent $H_2$ and gas samples were withdrawn at intervals for analysis. The most significant feature of the results was the long time necessary for equilibrium to be established even with the relatively large quantity of cells used. It can be seen from Table II, that equilibrium was not reached after 33 h although it appeared to be reached after 72 h in experiment 3.

Table II. Measurement of exchange reaction catalysed by *E. coli*

| Expt. No. | Rate ml $H_2$/h/mg dry wt. M.B. present | H | Atom % Deuterium Experimental i.e. *E. coli* present | Control i.e. no. cells present |
|---|---|---|---|---|
| 1 | 1·26 | 3 | 0·075 | 0·062 |
| | | 6 | 0·08 | 0·061 |
| | | 9 | 0·161 | |
| | | $10\frac{1}{2}$ | 0·129 | 0·066 |
| | | 12 | 0·138 | 0·066 |
| | | 27 | 0·215 | 0·071 |
| 2 | 1·25 | 24 | 0·328 | |
| | | 27 | 0·36 | |
| | | 30 | 0·328 | |
| | | 33 | 0·410 | |
| | | Days | | |
| 3 | 1·85 | 3 | 0·465 | 0·186 |
| | | | 0·452 | |
| | | 7 | 0·476 | 0·173 |
| | | | 0·483 | |

The experimental flasks contained 8 mg dry wt. cells suspended in 20 ml 0·2 M phosphate buffer pH 7·2. Initial gas phase 100 per cent $H_2$. Atom percentage deuterium in gas phase measured by mass spectrometry. Heavy water was added initially to the liquid phase to give 1 per cent (v/v) final concentration. The control flasks contained no bacteria but heavy water at the same concentration as in the experimental flasks. The rate of hydrogenation of methylene blue was determined separately with 100 per cent $H_2$ gas phase; methylene blue, 0·008 M final concentration, temperature 37 °C.

## Discussion

In these, admittedly rather limited, experiments we have been unable to demonstrate any discrimination between hydrogen and

deuterium in the hydrogenation reaction catalysed by bacteria. Franke and Mönch (1948) also observed that deuterium was taken up at the same rate as pure hydrogen when *E. coli* catalysed the hydrogenation of fumaric acid to succinic acid. Nevertheless, the succinic acid formed when deuterium was taken up contained little deuterium, i.e. the hydrogen added to the fumaric acid was mainly light hydrogen. Farkas and Schneidmesser (1947) obtained a similar result. The most probable explanation of these facts is that the 'activated hydrogen' on the catalyst surface is exchanged more rapidly with light hydrogen in the surroundings than it is used on the hydrogenation reaction. If the rate of hydrogenation is limited by the rate of reduction of the acceptor rather than by the rate of activation of hydrogen or deuterium no isotope effect would be expected and the acceptor will be mainly reduced with light hydrogen.

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# Production and Isolation of the Antibiotic, Oligomycin

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*Summary.* Production of oligomycin has been studied in fermentations ranging from 100 ml in shaken flasks to 1000 gal in agitated and aerated tanks. The yields ranged from nothing to 3675 μg per ml. The main factor favouring the production of oligomycin was a medium giving good growth and supplying an abundance of slowly utilized energy. Media meeting these requirements contained yeast extract and/or molasses for growth and lard oil for energy. Glycerol, glucose or starch as the main source of carbon gave poor results. A pH close to 7 favoured oligomycin production. Considerable organic nitrogen was synthesized and excreted by the mycelium during oligomycin production.

Under suitable conditions practically all of the oligomycin remained in the mycelium. Extraction of the mycelium with acetone, concentration of the extract, removal of oily material with a low-boiling hydrocarbon, decolourization and final crystallization from methanol or ethanol were the main steps in the recovery process. From pilot plant and tank fermentations about 4500 g of crystalline oligomycin have been prepared.

Three oligomycins, A, B and C were found by paper chromatography in even well-crystallized oligomycin. In different lots the proportions varied from 10–75 per cent A, 4–90 per cent B and 0–36 per cent C. High yields and high percentages of A and C were obtained on the neutral, lard-oil medium. Low yields and high proportions of B were given by the glycerol, low-pH medium.

## Introduction

Oligomycin is a complex of substances with antifungal and antinematode activity. It is produced by a streptomyces species

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† With the DuPont Company, Wilmington, Delaware.
‡ With Abbott Laboratories, North Chicago, Illinois.



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similar to *Streptomyces diastatochromogenes* and was first reported by Smith, Peterson and McCoy[1] in 1954. It was recovered from the culture filtrate in a yield of about 30–60 μg/ml. Paper chromatography of the beer showed the presence of two components A and B, one of which (B) however was lost during purification. Halliday[2] found that under suitable conditions oligomycin was retained in the mycelium, which greatly facilitated the isolation of the antibiotic. Modification of the medium increased the yield to about 235 μg/ml. Both oligomycin A and B were found in the mycelium extracts and crystals. Visser[3] continued the study of factors affecting the production of oligomycin. By means of lard oil and other nutrients, control of pH and selection of cultures, yields of up to 1450 μg/ml were obtained. In addition to A and B, a third component, oligomycin C, was found to be present in both beers and crystalline product. Several hundred grams of the oligomycin complex were prepared. Davis[4] and Winter[5] further studied the influence of media and culture selection on the total yield and ratio of the oligomycins. The main factor for yield improvements was found to be the composition of the culture medium rather than the culture. Isolates selected after ultraviolet irradiation did not show appreciable improvement of the oligomycin yield.

Working with various crystalline preparations, Marty[6] developed a column chromatography method for the quantitative separation of the three components in amounts sufficient to allow determination of their biological activity. His findings were that in general the antifungal activity of the oligomycins followed the same pattern, A > B > C, as their toxicity for mice by intraperitoneal administration (LD 50 was respectively 1·5, 2·9, 8·3 mg/kg body weight). Masamune *et al.*[7] expanded the column chromatography of the crystalline complex to larger quantities of substance and obtained homogeneous crystalline preparations of oligomycins A, B and C. Elementary analyses and molecular weight determinations gave the following formulae: A, $C_{24}H_{40}O_6$; B, $C_{22}H_{36}O_6$ and C, $C_{28}H_{46}O_6$. Although they differ in solubility, melting point, optical activity and elementary composition, their ultraviolet and infrared spectra are much alike, indicating that the structure of the three oligomycins is quite similar.

Leben *et al.*,[8] Zaumeyer[9, 10] and Szkolnik and Hamilton[11] have

reported some favourable results in the treatment of plant diseases. Phelps[12] has made an extensive study of oak-wilt and obtained considerable success in its treatment with oligomycin. Sladky[13] found that oligomycin was taken up by plants through root and leaf systems.

In the present paper, fermentation and extraction data obtained on the production of oligomycin will be reported.

## Experimental Procedure

### *Microbiological Assay*

In the first part of the work a strain of *Aspergillus fumigatus* was used, but it was later discontinued in favour of *Glomerella cingulata*. Both organisms were used with the paper-disc agar diffusion assay. *A. fumigatus* was carried on slants of the following medium: Difco malt extract 4 per cent, Difco peptone 0·1 per cent, Difco agar 1·5 per cent in tap water. The pH was adjusted to 6·0 with NaOH. The inoculated slants were incubated for 4 days at 30°C, by which time good sporulation had taken place. Assays were run in sterilized Pyrex baking dishes (8½ in. × 11½ in.), covered with a glass plate superficially sterilized with alcohol or acetone. The assay medium contained Difco peptone 0·5 per cent, Difco yeast extract 0·1 per cent, glucose 0·1 per cent, $KH_2PO_4$ 0·1 per cent, NaCl 0·5 per cent, Difco agar 1·5 per cent in distilled water. Three portions of the assay medium of 80, 80 and 60 ml respectively were melted, cooled and held temporarily in a 48°C waterbath. The first layer of 80 ml was poured into the dish and allowed to solidify. The second portion of 80 ml was then inoculated with 8 ml of a spore suspension of *A. fumigatus* or *G. cingulata*, standardized to give 50 per cent transmission in an Evelyn colorimeter. After thorough mixing, the seeded agar was poured on to the first layer and allowed to set. Then the unseeded third layer (to prevent spreading of the mycelium) of 60 ml was poured on to the second layer and also allowed to set. The dish was then covered and placed at 4°C until the preparation of the paper discs containing the antibiotic was completed. Schleicher and Schuell paper discs No. 5271—M, ½ in. diameter, were dipped in standard and test solutions and allowed to dry on two layers of filter paper. Duplicate discs were prepared and



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placed diagonally on the agar surface to eliminate the effect of differences in depth of the agar. The dish was placed at 4°C for 1–2 h to allow diffusion of the antibiotic, and then incubated at 30°C for 16–20 h. By this time well defined inhibition zones had formed. Their diameter was measured to 0·25 mm and a standard curve drawn on semi-logarithmic paper. The standard curve ranged from 8–64 μg/ml for *A. fumigatus* and 0·5–6 μg/ml for *G. cingulata* with zones measuring from 16–25 mm in diameter. The oligomycin content of unknown samples was calculated from their zone diameters and dilutions used. The accuracy of the assay was about 14 per cent when duplicate discs were used. The arbitrary standard was a repeatedly recrystallized material (No. V4) obtained from one of the 40-gal pilot plant fermentations. It was found later that it contained 67 per cent oligomycin A, 13 per cent B and 20 per cent C. *A. fumigatus* was discontinued because some strains of this organism are pathogenic to man. *G. cingulata* besides being harmless is more sensitive to oligomycin but requires a longer incubation (36–48 h).

### *Preparation of Fermentation Samples*

Thirty ml of fermented medium were filtered on a 4 cm Hirsch funnel and the mycelial pad washed once with 5 ml of distilled water. The pad was then quantitatively transferred to a 25 × 200 mm test tube. Five ml of acetone were added and the pad broken up with a glass rod. Then 20 ml of acetone were added, the mycelium was resuspended and extraction continued at room temperature for 1 h. The volume was then adjusted to 30 ml and the proper dilutions made in 95 per cent ethanol.

### *The Oligomycin Culture*

Several strains of the oligomycin-producing culture were used. The parent culture was the same as that of Smith *et al.*[1] and Halliday.[2] Following a method given by Thorne and Peterson,[14] Visser[3] plated this culture on oatmeal agar containing different levels of crystalline oligomycin complex, 20 per cent A and 80 per cent B. A total of 14 colonies were picked from media containing 30 and 40 μg of oligomycin per ml of medium. Of these, the cultures

30–1 and 30–6 were the best producers in the preliminary screening and were used in most of the later work. Stock cultures were carried in soil. Spore slants were made on oatmeal agar. Thirty grams of oatmeal were boiled with 1 l. of tap water and the suspended solids removed by filtration through cheese cloth. The filtrate was made up to 1 l. and 20 g of agar were added. The pH was adjusted to 6·8. Slants were made up in the usual way and sterilized for 30 min at 120°C. After seeding with a loop of soil stock, the slants were incubated for 5 days at 30°C, at which time they were well sporulated. They were stored at 4°C until used but for not longer than 2 months.

*Shake Flask Fermentation Procedure*

To prepare inoculum, 500-ml Erlenmeyer flasks were made up with 100 ml of the following medium: $(NH_4)_2HPO_4$, 0·4 per cent; $KH_2PO_4$, 0·2 per cent; $MgSO_4.7H_20$, 0·1 per cent; Difco yeast extract, 0·3 per cent; cornstarch, 1 per cent. Lard oil, 1 per cent, was added after adjusting the pH to 6·8. The flasks were then sterilized for 30 min at 120°C and cooled. Spores from slants were transferred by loop into the seed flasks, which were incubated for 48 h at 30°C on a rotary shaker turning at 250 rev/min with a 2-in. stroke. After growth had been well started, 0·5 per cent of $CaCO_3$ was added aseptically. This culture was used to inoculate the fermentation shake flasks at a rate of 5 per cent and these were likewise incubated at 30°C on a rotary shaker.

Two types of media were used in flask and tank fermentations. The composition of these media is given in Table I. Medium I was patterned after that of Halliday but in addition contained yeast extract or a substitute to give better growth of the micro-organism. Medium I had the disadvantage that the pH dropped rapidly early in the fermentation with resultant poor growth and oligomycin yields. Addition of 0·5 per cent $CaCO_3$ improved the pH picture and growth but it never gave high yields.

A simpler but higher-yielding medium, II, was obtained by omitting the calcium, iron and manganese salts (found unnecessary), replacing glycerol with lard oil and adjusting the pH to 6·8 before sterilization. Modifications of this medium are given in connection with the fermentation data. Essentially the same

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Table I. Composition of media used in the production of oligomycin

| Medium I | | Medium II | |
|---|---|---|---|
| Components | % | Components | % |
| Glycerol | 2·0 | Cornstarch | 0·2 |
| Yeast extract or substitute | 0·3 | Lard oil | 2·0 |
| $(NH_4)_2HPO_4$ | 0·4 | Yeast extract or substitute | 0·3 |
| $K_2HPO_4$ | 0·2 | $(NH_4)_2HPO_4$ | 0·4 |
| $MgSO_4.7H_2O$ | 0·1 | $K_2HPO_4$ | 0·2 |
| $CaCl_2.2H_2O$ | 0·04 | $MgSO_4.7H_2O$ | 0·1 |
| $FeSO_4.7H_2O$ | 0·002 | Silicones DCA | 0·02 |
| $MnSO_4.7H_2O$ | 0·001 | $CaCO_3$* | 0·5 |
| Silicones DCA | 0·01–0·03 | | |
| $CaCO_3$* | 0·05 | | |

* The $CaCO_3$ was usually sterilized separately and added when the pH reached 5·8, about 24 h after inoculation.

medium, but in some cases with less oil, was used to produce the inoculum. The quantity of inoculum used was about 5 per cent v/v of the fermentation medium.

### *Results in Shaken Flasks*

Many fermentations were run under different conditions, but only the most significant will be reported. A comparison of carbon sources for oligomycin production is given in Table II.

Table II. Comparison of several carbon sources for oligomycin production in flasks*

| Carbon Source | pH at | | | Oligomycin in mycelium at 96 h, μg/ml |
|---|---|---|---|---|
| | 24 h | 72 h | 96 h | |
| 2% glycerol | 6·2 | 7·4 | 7·8 | 110 |
| 2% glycerol + 1% lard oil | 6·2 | 7·0 | 7·2 | 370 |
| 2% glucose | 5·2 | 7·6 | 7·9 | none |
| 2% glucose + 1% lard oil | 5·2 | 7·0 | 7·2 | 270 |
| 2% cornstarch | 6·2 | 7·7 | 7·9 | none |
| 2% cornstarch + 1% lard oil | 6·0 | 6·9 | 7·1 | 520 |
| 4% cane molasses | 5·8 | 7·8 | 8·1 | none |
| 4% cane molasses + 1% lard oil | 5·8 | 7·0 | 7·4 | 270 |
| 2% lactose | 5·9 | 7·5 | 7·8 | 100 |
| 2% lactose + 1% lard oil | 6·3 | 6·9 | 7·3 | 390 |
| 0·2% glucose + 2% lard oil | 6·6 | 7·0 | 7·1 | 705 |

* Basal medium: $(NH_4)_2SO_4$, 0·4%; $KH_2PO_4$, 0·2%; $MgSO_4.7H_2O$, 0·1%; Difco yeast extract, 0·3%. $CaCO_3$, 0·5%, was added after growth was well started. Culture M 248 was used.

The highest yield (705 μg/ml) was obtained with a small amount of glucose (0·2 per cent) and liberal amounts (2 per cent) of lard oil. Glycerol or carbohydrate alone gave little or no oligomycin. The reason for the poor yields with carbohydrate might be due to the heavy early growth, inadequate energy for the later stages of fermentation and a high pH, which tends to destroy the oligomycin already produced. With carbohydrate and lard oil there was adequate growth and also sufficient energy for production of oligomycin. The pH remained in the range of optimal oligomycin production and prevention of autolysis of the mycelium.

A more detailed study of the oil requirements was made with medium II in which levels of oil were varied from 0 to 3 per cent. The best yields (around 1000 μg/ml) were obtained with levels of 1·5–2·5 per cent. No oligomycin was obtained with less than 1 per cent oil and in such cases the pH rose to 8 or higher. With 1 per cent or more lard oil the pH remained between 7·0 and 7·4.

As yeast extract is too expensive a nutrient for use in large-scale fermentations several substitutes were tried as shown in Table III. The most satisfactory material was cane molasses at

Table III. Comparison of substitutes for yeast extract in flask fermentations*

| Material | % | Final pH | Time of peak yield, h | Peak yield, μg/ml |
|---|---|---|---|---|
| Difco yeast extract (control) | 0·3 | 7·0 | 109 | 1455 |
| Corn steep liquor | 0·5 | 5·5 | 98 | 110 |
| Soybean meal | 0·3 | 6·1 | 98 | 155 |
| Cane molasses | 0·2 | 7·2 | 109 | 320 |
| Cane molasses | 0·6 | 7·1 | 109 | 1120 |
| Cane molasses | 1·0 | 7·1 | 109 | 1195 |
| Cane molasses<br>yeast | 0·6<br>0·3 | 7·1 | 120 | 1600 |

* Basal medium B and culture 30–1 were used.

a level of 0·6–1 per cent, but the yields were usually somewhat lower than those obtained with yeast extract in the same run. A combination of cane molasses and nutritional dried yeast gave the highest yield, 1600 μg/ml.

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### *Pilot Plant Fermentations*

On the basis of the results obtained in shaken flasks, fermentations were run on a pilot plant scale. Two stainless steel tanks of 60-gal capacity were used for this purpose. They were equipped with an aerator-agitator system, temperature control, a foam detector, an automatic lard oil feed, an inoculating port, and a sampling line. About 30 gal of medium were placed in the tank and sterilized by running steam into the medium until the temperature reached 120°C and held for 45 min. The volume of medium after sterilization, cooling, and inoculation was around 40 gal. The inoculum was grown in 30-l. aerator-agitator fermentors on medium II with cornstarch increased to 1 per cent and lard oil decreased to 1 per cent. $CaCO_3$ was added as mentioned before. About 8 l. of a 48-h culture were transferred aseptically through a hose line into the tank. During fermentation the aeration rate was held at 0·5–0·7 v/v of medium per minute. The agitator was run at 150–170 rev/min and the temperature was maintained at 28°C. After growth was well started, around 18–24 h, a slurry of sterilized calcium carbonate equivalent to 0·5 per cent of $CaCO_3$ based on the volume of medium was added. In some runs the $CaCO_3$ was added in the make-up medium. The aim was to run the fermentation as close to pH 7·0 as possible (most fermentations tended to become alkaline) and, if the pH rose above 7·0, more sterilized lard oil was added. Occasionally acidity developed (e.g., pH 5·5) and then more sterilized $CaCO_3$ slurry was run in. The usual time of fermentation was 96–120 h. About 25 tank fermentations were run, the most successful of which are reported in Table IV. Early in the work, run 1 was made with the parent culture, M 248, in the synthetic medium of Halliday. The yield was about the same in magnitude as he reported. In run 3 growth was improved by adding 0·3 per cent yeast extract and acidity was corrected by adding $CaCO_3$. These changes increased the yield considerably but they were still rather low for preparative purposes. After the flask results with medium II were obtained (Table II) this medium was tried in tank runs. Besides the change to a lard oil medium the new strains of the oligomycin-producing culture were also used. The data in Table IV show

Table IV. Production of oligomycin in pilot plant fermentations

| Run | Medium | Culture | pH | Oligomycin | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | h | μg/ml | % A | B | C |
| 1 | I | M 248 | 4·2 | 83 | 130 | 20 | 80 | 0 |
| 3 | I | M 248 | 6·3–7·4 | 83 | 204 | 50 | 50 | 0 |
| 4 | II | 30–1 | 6·6–7·1 | 94 | 1030 | 62 | 10 | 28 |
| 7–23 | II | 30–6 | 4·9–7·2 | 70–120 | 600–1170 | 60–75 | 4–18 | 14–36 |
| Average of 7 | II | 30–6 | 5·0–7·0 | 100 | 930 | 70 | 11 | 19 |
| Average of 2 30-litre fermentors | II | 30–6 | 5·1–6·9 | 163 | 1100 | not determined | | |

[a] In run 3 medium I was supplemented with 0·3% yeast extract at the time of make up and with 0·5% $CaCO_3$ after 24 h of fermentation. In runs 7–23, cane molasses replaced the cornstarch and yeast extract of medium II. The pH values are for the period of oligomycin production—from about 40 h to the end of the run. All fermentations were made at 28 °C.

that around 1000 μg/ml were obtained. Two runs in small 30-l. fermentors gave about the same yields. The two derived cultures showed no significant differences in yield. They cannot be compared with the parent culture since the latter was not run in the new medium. In flasks, Winter[5] found that M 248 likewise gave high yields on the oil medium, so the results should be attributed to the medium and not to the culture.

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*Changes in Nitrogen During Fermentation*

A shaken flask run in medium II was made in order to follow the changes in nitrogen in mycelium and filtrate during fermentation. Approximately every 24 h one flask of the series was removed and analyzed. The results are shown in Fig. 1.

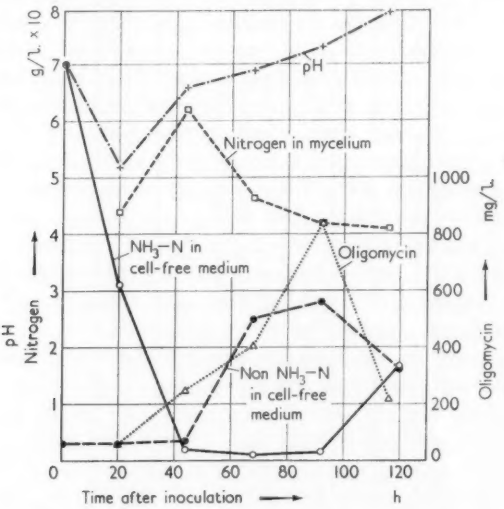


Fig. 1. Changes in nitrogen during fermentation.

Ammonia nitrogen was taken up rapidly and at 44 h practically all of it had been utilized. Weight of mycelium was not obtained in this fermentation, but in other runs the weight ranged from 10–15 g of dry weight per litre. At 44 h nitrogen in the mycelium was at a maximum. Excretion of nitrogen then began and in the next 24 h about 25 per cent of the mycelial nitrogen was lost to the medium. Presumably this was organic nitrogen as there was no increase in the ammonia nitrogen. During the next 24 h not much change occurred in the nitrogen picture but between 92 and 116 h the organism began to use the carbon of the organic matter and, the nitrogen of the compounds being apparently not needed, ammonia nitrogen accumulated. The pH rose rapidly during this

time and simultaneously about 75 per cent of the oligomycin was destroyed. The metabolic picture indicates a lack of carbon for energy purposes. In a tank run, development of this unfavourable situation was prevented by feeding lard oil at such a rate as to keep the pH close to 7·0. To date the pH of the medium has proved to be the best guide for operational purposes. Weight and nitrogen content of the mycelium has varied widely depending mainly on the nitrogen and lard oil content of the medium.

Excretion of organic nitrogen was roughly parallel to oligomycin formation although the latter contains no nitrogen and, if the pH stays around 7·0, remains in the mycelium. Nitrogen excretion seems to be a phenomenon common to many kinds of micro-organisms and is not necessarily linked with antibiotic formation.[15]

### *Isolation of Crystalline Oligomycin*

The mycelium was separated in a filter press (no filter aid was needed), washed with water until the effluent became colourless, and the excess water expelled with air. The filter cake was removed from the press and suspended in acetone to give a final concentration of 70 per cent (1·8 l. per kilo of press cake). This concentration of acetone gave good extraction of oligomycin and a low level of impurities. After standing for a few hours at room temperature, the mycelium was filtered off and again extracted with 70 per cent acetone. The two extracts were combined and concentrated in a vacuum still until serious foaming developed—usually to about 40 per cent of the original volume. The turbid liquid was then extracted twice with 0·25 volume of ethylacetate. The two extracts were combined and evaporated to about 25 per cent of their volume. Water separated out during this operation and was discarded after washing twice with ethylacetate. The washings were added to the main portion and water was removed by drying over anhydrous $Na_2SO_4$. The dried extract was concentrated to a syrup which was then poured slowly with stirring into 25 volumes of cold petroleum ether (b.p. 65–70°C). Most of the oligomycin precipitated while unused lard oil and other fatty impurities remained in the petroleum ether. The precipitate was filtered off by means of filter aid (Johns Mansville, Celite 545). The cake was washed with 0·1 volume of



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petroleum ether and was then spread out to dry. The dry powder contained about 25 per cent oligomycin and was suspended in 5 times its weight of 95 per cent ethanol. About 1 g of Darco G 60 per 100 ml of alcohol was added to decolourize the solution and after 1 h carbon and filter aid were removed by filtration. The cake was washed twice with small portions of 95 per cent ethanol. The washings and main filtrate were then concentrated in a vacuum to about 15–20 per cent of their volume, warmed to 40°C and water added to the incipient cloudiness of the precipitating oligomycin. The liquid was seeded with crystals of oligomycin and allowed to cool slowly. The antibiotic crystallized out on standing. The over-all recovery of oligomycin based on the content in the mycelium in the best runs has been about 50 per cent and in the poorest about 25 per cent.

The main losses in isolation were: 20 per cent remaining in the mycelium, 20 to 50 per cent in the petroleum ether and 5 to 10 per cent in the mother liquors. More careful extraction of the mycelium removed more of the oligomycin but little success was achieved in reducing the loss of oligomycin in the petroleum ether. This liquid seemed to contain a waxy material as well as unused oil. Undoubtedly the above method of isolation could be improved by careful, systematic study. The main purpose of this work was not to obtain an especially high recovery but to provide sufficient crystalline material to effect a separation of the three components and to make possible a study of their physical, chemical and biological properties. From the many pilot plant runs about 300 g of crystalline oligomycin complex have been prepared.

### *Preparation and Properties of Oligomycins A, B and C*

The components in the crystalline preparations have been separated and quantitatively determined by both paper and column chromatography.[1, 7] The results reported here are by paper chromatography and given in columns 7 to 9 of Table IV. The ratios ranged from 20 per cent A, 80 per cent B and no C in run 1, to 60 per cent A, 4 per cent B and 36 per cent C in run 23. From these data it seems that a glycerol medium gives a low pH, low yields and a high percentage of B. A lard-oil medium gives a

neutral pH, produces appreciably higher yields made up largely of A, a considerable percentage of C and usually, though not always, a small proportion of B.

### *Production of Oligomycin in Tanks*

Four lots of oligomycin were prepared in the laboratories of Chas. Pfizer and Co., Inc. by the methods described in the following sections.

#### *Fermentation*

Spores of the oligomycin-producing culture were introduced into 3-l. Fernbach flasks containing 1000 ml/flask, and incubated with shaking at 30°C for 24 h. Four litres of this inoculum were then transferred into a stainless steel seed tank containing 150 gal of broth. The tank was aerated at a rate of 1 v/v of medium and agitated at 1500 rev/min by a 4-blade pitched propellor. The fermentation temperature was closely controlled at 28°C. At the end of 48 h, the broth generally contains a sufficient amount of mycelial growth and is transferred into the final fermentor stage. The fermentor was run at an air rate of 0·5 v/v of medium and agitated with a curved turbine at 150 rev/min. The temperature was maintained at 28°C for approximately 72 h or until maximum potency was obtained.

Cultures M 248 and its variant 30–1 and media I and II with some slight changes were used. Distillers' solubles, 0·3 per cent. were substituted for yeast extract in medium I and 1 per cent cane molasses for the yeast extract in medium II. The same medium was used in both inoculum and fermentor. To reduce foaming silicone DCA was used with medium I at a level of 0·01 per cent and at 0·02 per cent with medium II.

The microbiological assay was run as already described with either *A. fumigatus* or *G. cingulata* as test organisms and crystalline oligomycin as the standard.

1. *Broths with Biopotency of* 150–200 *μg/ml.* The recovery of a 2000-gal batch of whole broth having a biopotency of 150–200 μg/ml is described below. Slight modifications of this recovery are necessary for broths with higher and lower biopotencies.

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The whole broth was filtered on an Oliver rotary vacuum filter using a wash rate of about one-tenth the filtration rate; the filtrate was discarded. The mycelium was repulped in 400 gal of acetone at room temperature for 2 h. The slurry was then allowed to settle, and the supernatant extract was first decanted, and then clarified through a pressure filter. The repulping, decanting and filtration operation were repeated twice using 250-gal portions of acetone. The spent mycelium sludge which usually retained less than 5 per cent of the activity was then discarded. The three acetone extracts were combined and concentrated at 25–35°C to about 25 gal. At this point the acetone content of the extract had been removed and the oligomycin, being only slightly soluble in water, precipitated. The slurry was filtered on a small Nutsche pressure filter, and the resulting filter cake was vacuum dried at 40°C.

The crude oligomycin was slurried in hexane (2 ml/g crude) at room temperature for 15–20 min to remove oil, then filtered, washed with hexane until the filtrate appeared free of oil, and vacuum dried at 40°C.

The oil-free crude was dissolved in methanol (20 ml/g oil-free crude) at 40–45°C and the resulting solution, following carbon treatment (0·0–2 g Darco G–60 g oil-free crude) and filtration, was concentrated at 20–25°C to 10 ml/g oil-free crude; crystallization occurred at this point. An equal volume of water was added continuously over a 30-min period to complete the crystallization. The slurry was then stirred an additional 30 min and filtered. The crystals, which were just off-white in colour, were vacuum dried at 40°C.

The oil-free crude was also recrystallized by cooling the carbon-heated methanol solution to 5°C, adding 0·75 volumes of water continuously over a 6-h period, and keeping the slurry at 5°C for an additional 10–12 h before filtering. This method reduced the biopotency of the mother liquor (from 250 to 100 μg/ml), but enhanced the tendency of the coloured impurities to precipitate with the oligomycin.

2. *Broths with Biopotency above* 200 *μg/ml.* With broths of biopotency greater than 200 μg/ml, the acetone repulp of the mycelium cake was modified to assure essentially complete extrac-

tion of the oligomycin. For example, the mycelium from 2000 gal of whole broth with a biopotency of 600–700 μg/ml, was repulped twice in 650 gal of acetone and twice in 275 gal of acetone. The remainder of the recovery was unchanged.

3. *Broths with Biopotency below* 150 *μg/ml.* When broths with biopotencies less than 150 μg/ml were processed, the crude oligomycin sometimes failed to precipitate from the concentrated extract. Two methods were employed to force the antibiotic from solution. In the first, the concentrate was cooled to about 5°C, 0·6 volumes of methanol were added continuously over a 2-h period, and the slurry was then stirred an additional 10–12 h at 5°C and filtered; biopotency of the mother liquor was 50–100 μg/ml. In the second, 0·1 volume of hexane was added to the concentrate at room temperature, the mixture was stirred 20–30 min, and the solids, which tended to collect at the hexane–water interface, were then filtered and washed with hexane until free of oils. This latter method, although eliminating the hexane repulping of the dried crude, resulted in considerable loss of activity in the hexane and water filtrate layers; the hexane layer had a biopotency of about 5000 μg/ml, while the water layer contained about 1500 μg/ml.

*Fermentation Results*

A summary of 11 fermentation runs, combined to give the several lots of product, is presented in Table V. A remarkable increase in fermentation yield was obtained starting with run 7;

Table V. Summary of tank fermentations

| Batch No.* | Age, h | Oligomycin, μg/ml | Recovery, % | Lot No. |
|---|---|---|---|---|
| 1 and 2 | 68, 73 | 60, 100 | 30 | 1843–42–5 |
| 3 and 4 | 71, 72 | 110, 70 | 45 | 1543–117–1 |
| 5 and 6 | 72, 72 | 90, 180 | 50 | 1543–134–1 |
| 7 to 10 | 72 to 87 | 1010 to 3675 | no data | ? |
| 11 | 69 | 615 | 80 | 2138–96–1 |

* Nos. 1–6 were run with culture M 248 in medium I in 2000-gal tanks. In nos. 7–10 culture 30–1 and medium II were used in 150-gal tanks. In no. 11 the culture was 30–1 and the volume was 1000 gal. No. 11 was badly contaminated.

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this was associated with changes in culture, medium and equipment.

The initial 6 runs were made with Culture M 248 and a medium based on glycerol and distillers' solubles. Yields ranged from 60–180 μg/ml in 72 h.

The combination of a new culture No. 30–1 and a new medium developed at the University of Wisconsin, was next evaluated in the plant. Runs 7 to 9 ranged from 1010 μg/ml to 1280 μg/ml, thus checking previously reported data. Run 10 made under the same operating conditions, yielded 3675 μg/ml. No obvious reason for this very high result could be determined. Run 11 although badly contaminated still gave 615 μg/ml.

*Recovery of Oligomycin*

A summary of the recovery data is presented in Table VI. For broths fermented with the old culture and old medium, containing a biopotency of 150 μg/ml, the recovery yield to finished

Table VI. Summary of recovery processing

| Items | Oligomycin Content Batch 3 | 4 | 5 | 6 | 11 |
|---|---|---|---|---|---|
| Broth potency, μg/ml | 110 | 72 | 90 | 180 | 615 |
| Total activity, g | 1080 | 670 | 910 | 1390 | 2130 |
| Filtrate, g | 40 | 90 | 70 | 60 | 40 |
| Acetone extract, g | 1030 | 1240* | 780 | 1560 | 3370 |
| Spent mycelium, g | 60 | 30 | 50 | 106 | 60 |
| Crude precipitate, g | 400† | 420‡, § | 1190 | | 2160 |
| Mother liquor, g | 80 | 170 | 30 | | 4 |
| Hexane slurry, g | 420 | 200 | 1270 | | 1960 |
| Mother liquor, g | 10 | 10 | 10 | | 10 |
| Finished crystalline product, g | 820 | | 1280 | | 1700 |
| Mother liquor and carbon cake, g | 10 | | 10 | | 10 |
| Overall recovery, nearest 5% | 45 | | 50 | | 80 |
| Lot No. | 1543–117–1 | | 1543–134–1 | | 2138–96–1 |
| Ratio of oligomycin A:B:C | 16:84:0 | | 10:90:0 | | 58:23:19 |

* Presumably high bio-assay.
† Methanol added to concentrate to precipitate crude.
‡ Based on apparent biopotency of 1000 μg/mg per crude.
§ Hexane added to concentrate to precipitate crude oligomycin: no hexane repulp was necessary.
** Methanol–water recrystallization at 5°C.

goods was in the range of 45–50 per cent (runs 3 to 6). The only major loss of activity appeared to occur during the concentration of the combined extracts.

The second type of broth was produced with the new culture and new medium and assayed 615 μg/ml. Although the particular batch recovered was badly contaminated, an excellent recovery of 80 per cent to finished goods was obtained. Essentially no losses were encountered during the extraction and concentration steps. This was undoubtedly due to the considerably greater oligomycin concentration that was obtained in the acetone extracts. In all about 4500 g of crystalline oligomycin have been made.

One of the most interesting results of this study is the variation found in the proportion of the three components in the different lots of oligomycin. The first three lots were roughly 80 per cent A, 20 per cent B and no C. This composition was associated with the type of medium; the low energy (glycerol) medium produced only a small amount of oligomycin and this was predominantly B. The second, a high energy (oil) medium produced from 6 to 35 times as much oligomycin, made up largely of A, and small and about equal amounts of B and C. These results are in agreement with those reported for pilot plant fermentations in Table IV.

To date no obvious explanation can be given of why a high energy medium favours the development of A and C to that of B. Perhaps some reason may appear when more is known about the structure of the three oligomycins.

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# The Disintegration of Micro-organisms by Shaking with Glass Beads

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*Summary.* A new vibrator, commercially available as the *Sonomec* Wave-Pulse Generator, is described, in which micro-organisms may be disrupted by shaking with glass beads. The amplitude and frequency of vibration of the shaker may be varied independently by suitable linkages incorporated in the mechanism. A study has been made of the effect of several variables, particularly the height of the liquid column in the vibrating container, on the rate of disruption. A relation is shown to exist between the acoustic heating produced in the container by the passage of sound waves through the liquid and the rate of disruption of baker's yeast. It is considered that at most depths of the liquid column, the action is similar to that of other shakers, but at a certain depth of liquid, additional forces arise which greatly increase the rapidity of disruption. How these forces act is not known. Factors affecting the rate of disruption of baker's yeast, *Corynebacterium xerosis* and *Bacillus megatherium* have been studied and a comparison has been made of the fractions obtained by differential centrifugation of the cell dispersion and compared with similar preparations from the Hughes press.

Agitation with glass beads, or other small particles, was first described by Curran and Evans (1942) and is now a well established method of disintegrating micro-organisms, especially for preparing cell walls. A number of instruments have been used to perform the shaking operation, from laboratory flask shakers (Furness, 1952; Cooper, 1953) to the Mickle (1948) and Nossal (1953) shakers. Of these instruments, the Nossal shaker is the most efficient. However, these devices exhibit one or more of the following disadvantages, and this does not make them ideal for this purpose:

(1) Small amounts (25 ml) of material only may be treated at one time.



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(2) Lengthy treatment is necessary to obtain an acceptable percentage breakage.
(3) A high ratio of particles to micro-organisms is required.
(4) Mechanical unreliability.
(5) Inability to disintegrate micro-organisms in concentrated sucrose solutions.
(6) Dilute suspensions only can be used.

The present work describes a mechanical shaker which does not possess any of the disadvantages listed above, and is commercially available. The apparatus, known as the *Sonomec* Wave-Pulse Generator, was designed for the propagation of sound waves of variable amplitude and low frequency through liquids, the original oscillation being generated mechanically by a vertically-moving piston assembly. Some of the types of preparation which have been obtained are described, and the enzyme activity of the extracts and particle preparations recorded; these preparations are compared with the corresponding ones produced by use of the Hughes (1951) bacterial press.

## Methods

*Growth of organisms.* Baker's yeast was obtained locally, and was freshly bought for each experiment. *Corynebacterium xerosis* (N.C.T.C. 7243) was grown on agar in rectangular metal trays (15 in. × 10 in. × 2 in.) with close-fitting lids. Each tray contained 400 ml of the following medium, with agar–agar added to 2 per cent (w/v); 0·5 per cent (w/v) D-glucose (B.D.H. Analytical Reagent grade), 2 per cent (w/v) Tryptone (Oxoid Ltd.), 0·5 per cent (w/v) yeast extract (Oxoid Ltd.), and 2 per cent (w/v) potassium dihydrogen phosphate (B.D.H. A.R. grade), buffered to pH 7 with 5 N sodium hydroxide solution. Trays were inoculated from agar slants of the organism grown on the same medium, and were incubated overnight at 37 °C. The organisms were harvested by scraping them from the surface of the agar with a clean microscope slide, and suspending them in 0·07 M potassium phosphate buffer, pH 7. *Bacillus megatherium* was grown on a liquid medium of the same composition as the medium described above. Usually 8 l. of organisms were grown in a 10-l.

Pyrex bottle, equipped with a sparger through which a vigorous stream of sterile air was forced. Two hundred and fifty ml of organisms grown in the same medium were used as an inoculum. Growth of the 8-l. batch at 37 °C was complete in 18 h, and the cells were harvested and washed with 0·07 M potassium phosphate buffer, on the Sharples centrifuge. An average yield of organisms was found to be about 6 g wet weight per litre.

*Cell counting.* Total counts of intact cells were performed in standard bacterial counting chambers of 0·02 mm depth, and examined by phase-contrast microscopy. Each suspension was thoroughly shaken for 8 min on a 'Microid' laboratory flask shaker (Griffin and Tatlock Ltd.) before pipetting onto the slide. Yeast counts were always performed in duplicate and *B. megatherium* counts in triplicate: agreement in each case was better than ±3 per cent. The ratio of broken to unbroken cells was determined by a differential count by electron microscopy. Each count was performed in duplicate and an error of ±6 per cent was not usually exceeded.

*Electron microscopy.* Samples were taken from the material diluted for counting. A suitable volume, generally about 0·2 ml, was diluted in 1 ml of aqueous 1 per cent (w/v) osmium tetroxide and after 5 min was plated onto formvar covered grids by the blotting technique. Usually evenly spread fields were obtained with bacteria, but with aqueous suspensions of yeast it was found that all the cells and large cell fragments gathered together in clumps touching the copper mesh of the grid and leaving the centre of the field empty. Spraying the yeast by the method of Williams and Backus (1949) was not satisfactory and the method finally adopted was as follows. A drop of the fixed or unfixed yeast suspension was placed on a grid which was held close to a brass plate (1 cm wide × 6 cm long × 0·25 cm deep) supported at both ends on solid carbon dioxide (Cardice). The brass plate was wiped with hardened filter paper to remove frozen moisture and the grid held in close contact until the film of suspension was frozen. It was then released from the forceps onto the plate. Up to a dozen grids were prepared at one time on the brass plate which was then dried in a freeze-dryer with acetone–carbon dioxide and liquid air traps. The vacuum was let down gently and a wire connected to the brass plate in order to discharge static

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electricity which otherwise caused the grids to jump violently when the plate was moved. This method was unsuitable when sucrose was present, but usually it was found possible to make satisfactory fields from fixed preparations by the blot method, and to wash away the sucrose after drying in a stream of warm air. Electron microscopic examination was carried out in an Akashi Tronscope (Akashi Ltd., Japan) usually at magnifications from 600 to 10,000 diameters. Counting was carried out directly from the screen, and not from photographs. Usually at least 200 cells or cell fragments were counted on each grid. As far as possible only separate cells were counted and clumps containing more than four cells were omitted.

*Nitrogen* was determined by a micro-Kjeldahl method.

*Enzyme assays.* Succinic dehydrogenase was determined by following the reduction of 2:6 dichlorphenolindophenol at 6000 Å as described by Green, Mii and Kohout (1955). The reaction mixture contained 30 μmoles of potassium cyanide, 30 μmoles of potassium succinate, 3 μmoles of disodium ethylene diamine tetracetate, 60 μg dye, and enzyme solution to a total of 3·0 ml. The reaction was started by the addition of enzyme, and dye reduction determined against a blank cell containing water instead of substrate was followed in the Cary recording spectrophotometer. Activities were expressed as μmoles dye reduced/mg N/min.

*Lactic dehydrogenase* activity was followed by the same procedure as for the succinic dehydrogenase except that 30 μmoles of sodium lactate were used instead of succinate. Enzyme activity was expressed in terms of μmoles dye reduced/mg N/min.

*Fumarase* activity was determined by the reduction of optical density at 2850 Å. Cuvettes contained 1·5 ml 0·07 M potassium phosphate buffer pH 7, 30 μmoles sodium fumarate, and enzyme solution to a total volume of 3·0 ml. In the blank cuvette, the fumarate was replaced with an equal volume of water. The reaction was started by the addition of enzyme, and the reaction followed on the Cary recording spectrophotometer. Activities were expressed in terms of μmoles DPNH formed/mg N/min.

*Alcohol dehydrogenase* activity was measured at 3400 Å. Cuvettes contained 2·0 ml semicarbazide solution in 0·1 M glycine buffer pH 9·6 (0·75 mg semicarbazide HCl/ml glycine buffer), 0·9 ml water, 0·1 ml DPN (10 mg/ml) and 0·1 ml enzyme solution.

The reaction was started by the addition of 0·1 ml absolute ethanol to the experimental cuvette, and 0·1 ml distilled water to the blank, and the change in optical density followed in the Cary recording spectrophotometer. Activities are expressed as μmoles DPNH formed/mg N/min.

## Description of the Apparatus

The apparatus to be described is known as the *Sonomec* Wave-Pulse Generator (D.F. Developments Ltd., 2 Clement's Inn, Strand, London, W.C.2.). It comprises a 2 h.p. electric motor

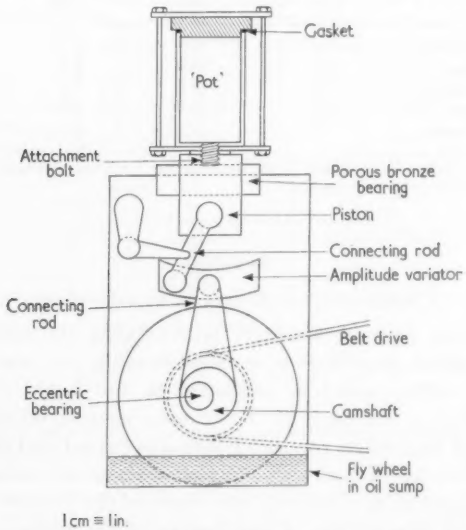


Fig. 1. Diagram of the *Sonomec* Wave-Pulse Generator. Scale is approximately 1 cm = 1 in. The 2 h.p. electric motor is not shown

coupled through an infinitely variable gear (range 3–1 up or down), and a flat-belt drive to a piston assembly. The piston assembly incorporates a linkage by means of which the amplitude of the piston movement may be varied within the limits 0–0·3 cm. The motor is geared such that the piston may be driven with a sinusoidal motion at frequencies from 25–200 c/sec. The whole

machine is enclosed in a large wooden container lined with sound absorbing fibre-glass, and may be operated whilst so enclosed. Metal 'pots' or containers of various sizes may be attached to the piston simply by screwing them into the threaded hole provided. The apparatus is shown in Fig. 1. A number of pots have been constructed for use with the machine: they are listed, together with their dimensions, in Table I.

Table I. Dimensions of 'pots' for *Sonomec* shaker

| Pot | Material | Internal diameter (cm) | Internal length (cm) | Lid depth (cm) | Available depth (cm) |
|---|---|---|---|---|---|
| I | Mild steel | 5·2 | 12·1 | 2·5 | 9·6 |
| II | Brass | 2·5 | 18·9 | 1·1 | 17·8 |
| III | Stainless steel | 2·5 | 9·2 | 0·4 | 8·8 |
| IV | Stainless steel | 2·2 | 25·9 | 0·75 | 25·15 |
| V | Perspex | 5·0 | 9·5 | 1·5 | 8·0 |

The 'available depth' is the length of the inside of the pot which is available for carrying liquid when the lid is fastened in position.

## Results

### *Conditions for efficient energy coupling*

It was clear from preliminary studies that the rate at which micro-organisms were broken in the vibrating pot, was related to the energy which could be imparted to the liquid (and beads) inside the pot, as indicated by the acoustic heating which occurred. It was found that when acoustic heating occurred and the contents of the pot became warm, more organisms had been disintegrated.

It was therefore decided to study some of the factors controlling the efficiency of coupling of the energy of the piston (and hence the pot) to the liquid inside the pot. The acoustic heating produced (measured by the increase in temperature, $\Delta t$) was taken as a measure of the energy transferred. The validity of this assumption was considered to be borne out by the results obtained.

*Effect of height of liquid column on $\Delta t$.* The metal pot was cooled in ice to 2–3°C and then filled to a suitable depth below the top of the pot with ice-cold water. This depth was measured accurately with an adjustable metal probe, connected through a

micro-ammeter to a 65 V dry battery. The other side of the battery was connected to the outside of the pot. The probe could be lowered by a rack-and-pinion mechanism with a precision of 0·001 cm measured on a drum scale. The difference (in cm) between the top of the pot and the surface of the liquid (when the micro-ammeter registered the passage of a current) could then be

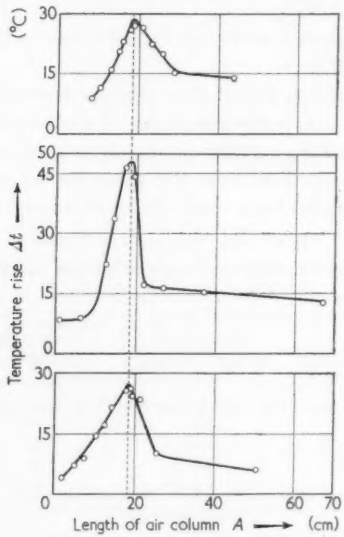


Fig. 2. Effect of length of air-column ($A$) on the acoustic heating ($\Delta t$) in pots I, II and IV. The conditions of the experiments were respectively: Pot I contained 50 g No. 12 ballotini beads: vibrated at 117 c/sec and with 0·3 cm amplitude for 75 sec. Pot II contained 8 g No. 12 ballotini beads: vibrated at 117 c/sec and with 0·3 cm amplitude for 120 sec. Pot IV contained distilled water without ballotini beads: vibrated at 117 c/sec and with 0·3 cm amplitude for 120 sec

obtained with precision. An initial reading of temperature was taken ($t_i$ °C) and the lid securely fastened to the top of the pot, which was then vibrated under defined conditions of amplitude, frequency and time. At the end of the time period, the machine was stopped, the lid removed from the pot, and the temperature of the contents measured immediately with a 0–50 °C mercury-in-glass thermometer ($t_f$ °C). The pot was then cooled again, another

depth of liquid selected, and the experiment repeated. The frequency of the vibration was checked stroboscopically and found correct to 2–3 per cent at 117 c/sec. The temperature difference $\Delta t$ $(= t_f - t_i)$ was then plotted against the depth of liquid in the pot. Alternatively $\Delta t$ was plotted against the length of the column of air enclosed between the surface of the liquid and the underside of the lid of the pot (i.e. depth as measured above minus depth of the lid).

The results of such a series of experiments, with pots I, II and IV are shown in Fig. 2.

It may be seen from Fig. 2 that the maximum temperature rise in the pot occurs at a particular value of the length of the enclosed air-column. That the relation is purely one of length, and not of volume, is shown in Table II, the data for which are assembled from experiments identical with the ones described.

Table II. Lengths and volumes of the liquid and air columns under conditions where $\Delta t$ is a maximum

| Pot | Length of liquid column (cm) | Volume of liquid column (ml) | Length of air-column (cm) | Volume of air-column (ml) |
|---|---|---|---|---|
| I | 7·75 | 165 | 1·85 | 39·3 |
| II | 15·95 | 78·5 | 1·85 | 9·1 |
| III | 6·95 | 34·1 | 1·85 | 9·07 |
| IV | 23·3 | 88·8 | 1·85 | 7·05 |

The critical length of the air-column, 1·85 cm, is called '$A_{max}$'.

The effect of a number of other factors on the temperature rise at the $A_{max}$ length of air-column, 1·85 cm, has been studied.

*Effect of frequency on $\Delta t$.* The depth of water in pot I was adjusted to 1·85 cm below the level of the lid ($A_{max}$) with the probe, and the amplitude of the vibration to 2 mm. The temperature rise ($\Delta t$) over a 2-min period was measured as described earlier. Fig. 3 shows the results of such an experiment: at low values of the frequency, $\Delta t$ is small but increases with increasing frequency, until a maximum value is reached at a frequency of about 150 c/sec, after which it falls sharply to low values. This variation of $\Delta t$ with frequency is not due to a variation in the

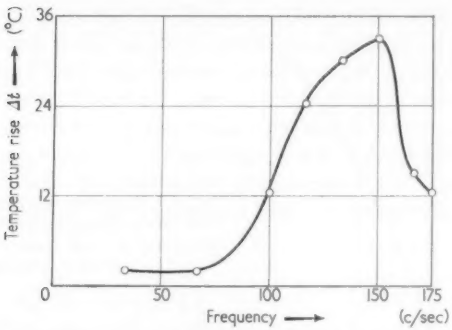


Fig. 3. Effect of frequency ($f$) on acoustic heating ($\Delta t$) in pot I: the amplitude of the vibration was 0·2 cm

$A_{max}$ value with frequency, as Table III shows. This experiment was conducted in the same way as the $\Delta t$/liquid depth experiments described above, at each frequency, and the $A_{max}$ value determined for each frequency from these curves. The error in the determination of $A_{max}$ is of the order of $\pm 0{\cdot}2$ cm, due to variation in the compression of the rubber sealing gasket in the lid of the pot. A new design of lid has reduced this error to the order of $\pm 0{\cdot}05$ cm.

Table III. '$A_{max}$' values determined from $\Delta t$/(length of air-space) curve for each frequency

| Frequency (c/sec) | 83·3 | 100 | 117 | 133·3 |
|---|---|---|---|---|
| '$A_{max}$' (cm) | 2·05 | 2·1 | 1·8 | 1·95 |

*Effect of amplitude of the vibration on $\Delta t$.* The effect of the amplitude of the vibration on the temperature rise in the pot is shown in Fig. 4. The experiment was performed in pot I. The height of liquid in the pot was adjusted to the $A_{max}$ value and 117 c/sec was used at the standard frequency. Conduction of the experiment was as before. Unlike the frequency effect, the amplitude effect may be shown to be the result of a variation in the $A_{max}$ value with amplitude, as shown in Fig. 5. The experimental technique was as before. The pot was cooled to

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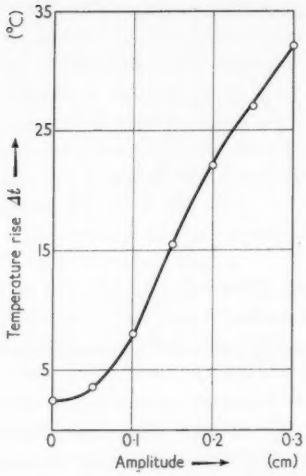


Fig. 4. Effect of amplitude of the vibration on the acoustic heating in pot I, at a frequency of 117 c/sec. Length of air-column was $A_{max}$

2°C, filled to an accurately measured depth by use of the probe, and was then vibrated at 117 c/sec, for 3 min in the case of the 0·15 cm amplitude and for 2 min for the 0·3 cm amplitude. Final temperatures were read immediately the lid had been opened. The temperature rise ($\Delta t$) was plotted against the length of the air-space $A$ for each amplitude.

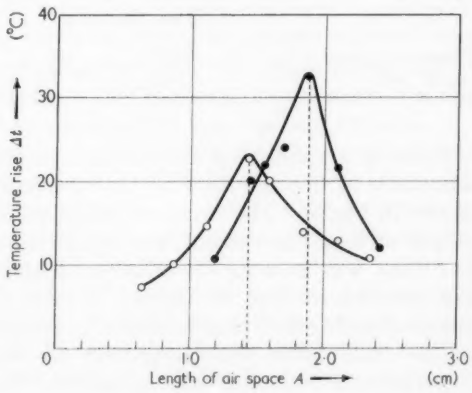


Fig. 5. Effect of length of air-space ($A$) on acoustic heating ($\Delta t$) at two different amplitudes of vibration. (○—○, 0·15 cm amplitude; ●—●, 0·30 cm amplitude)

*Effect of ballotini beads.* Many of the experiments described were performed with ballotini beads in the system in order to simulate more closely the system under working conditions. The absence of beads, however, does not significantly alter the $A_{max}$ value, as is shown in the following experiment with pot II. The pot, containing 8 g of No. 12 ballotini beads, was cooled in ice, and filled with ice-cold distilled water to a depth of 1·85 cm below the level of the lid. It was vibrated with an amplitude of 0·3 cm

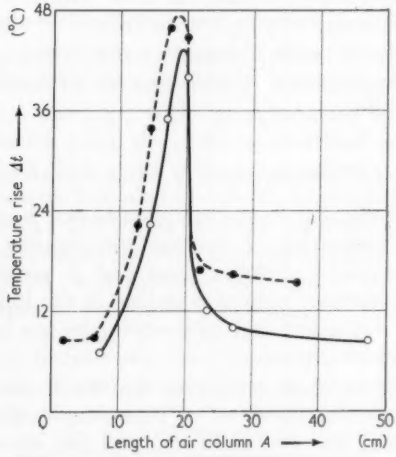


Fig. 6. Effect of ballotini beads on acoustic heating in pot II, at $A_{max}$ length of air-column. Pot vibrated at 117 c/sec and with 0·3 cm amplitude for 120 sec. (●– – –●, 8 g of No. 12 ballotini: ○—○, without ballotini)

for 2 min at 117 c/sec. $\Delta t$ was measured as before. The experiment was repeated, omitting the ballotini beads. The temperature rise ($\Delta t$) was plotted against the length of air-column $A$ in each case. The data are shown in Fig. 6. The increase in $\Delta t$ at the $A_{max}$ point may be accounted for by the decreased volume of the liquid when ballotini beads are present in the system.

*Miscellaneous effects.* Concentrations of from 0·1–1·0 M sucrose in water show an effect of depth of liquid on temperature rise similar to the one described without sucrose.

The effect of foaming of the liquid has caused difficulty in that the liquid held as a stable foam diminishes the depth of liquid in the pot (i.e. increases the value of $A_{max}$, the length of the air-space), and the system 'goes off tune' as it were. This effect is pronounced when micro-organisms are being disrupted, when a stable foam of denatured protein may be produced: usually an attempt is made to reduce the magnitude of the effect, by the addition of an inert antifoam such as tributyl citrate.

It is clear from these experiments that under closely defined conditions of frequency, amplitude and length of air-column enclosed, the efficiency of coupling of energy of vibration to the motion of the liquid/beads system is a maximum. Small variations from these optimum conditions can markedly reduce the efficiency of coupling.

*Rate of disintegration of micro-organisms*

Conditions for efficient energy coupling having been established, the rate of disintegration of two micro-organisms was studied. These micro-organisms, baker's yeast and *B. megatherium*, were chosen for convenience: ease of counting in the light microscope was considered important and both organisms are large in size.

The rate of disintegration of yeast was studied in the vibrating pot of the *Sonomec*, in an analogous manner to the temperature rise experiments. A suspension of yeast in phosphate buffer (of known counts/ml) was cooled in ice, and the ice-cold pot filled to the predetermined depth with the suspension and a suitable weight of ballotini beads. The lid was fastened down after an initial temperature reading had been taken, and the suspension vibrated under the selected conditions of time, frequency and amplitude. At the end of the period the pot was opened and the temperature of the contents measured. A sample was removed for total counts of intact yeast remaining and for differential counts of broken organisms. This differential count proved necessary as it was found that many organisms which appeared intact under phase-contrast microscopy were in fact broken when examined under the electron microscope. After centrifuging a sample of the disintegrated material for 10 min at 6,000*g*, duplicate samples of the supernatant fluid were removed for nitrogen

estimation. A volume of phosphate buffer (equal to the volume of sample removed from the pot), was replaced in the pot, and the experiment repeated. The dilution effect was taken into account in the calculation of the results. From the data obtained for total counts and the ratio of broken to unbroken cells, these percentages could be derived:—percentage of cells remaining intact; percentage of cells completely broken (decrease in total count); and the percentage of 'cell-walls' i.e. cell-walls containing various amounts of cytoplasmic material.

*Rate of disintegration of yeast at $A_{max}$ value*

A 1:6 suspension of fresh baker's yeast in 0·07 M phosphate buffer was cooled in ice and pot II, containing 10 g of No. 12 ballotini beads, was filled with the suspension to 1·85 cm below the level of the lid, using the probe as described earlier. The pot was vibrated at 117 c/sec with an amplitude of 0·3 cm and 4·0 ml samples were removed at 1·5-min intervals for 6 min. The pot was cooled to 2°C in ice between each period of vibration. Counts were performed as previously described on a 1:50 dilution of the suspension, and nitrogen in solution was estimated on duplicate samples of the 6,000*g* supernatant. The results of such an experiment are shown in Table IV.

Table IV. Disintegration of yeast at time intervals in the *Sonomec*. Length of air-column, 1·85 cm ($A_{max}$); percentage breakage measured as described in the text

| Time (min) | Nitrogen in solution (%) | Cells disappeared (%) | 'Cell-walls' (%) | Intact cells (%) |
|---|---|---|---|---|
| 1·5 | 6·2 | 22·0 | 10·5 | 67·5 |
| 3·0 | 16·3 | 30·0 | 17 | 53 |
| 4·5 | 18·0 | 44·0 | 18·5 | 37·5 |
| 6·0 | 21·8 | 44·0 | 21 | 35 |

*Rates of disintegration of yeast at length of air-column greater than $A_{max}$*

The experiment was repeated exactly as above, except that the $A$ value was 6·4 cm (instead of 1·85). Time intervals were 3, 6,

12 and 24 min, and the final 12-min period was divided into two periods of 6 min each, with cooling in ice in between: the pot was not opened during this time. The data of such an experiment are given in Table V.

Table V. Disintegration of yeast at time intervals in the *Sonomec*. Length of air-column, 6·4 cm (not $A_{max}$); percentage breakage measured as described in the text

| Time (min) | Nitrogen in solution (%) | Cells disappeared (%) | 'Cell-walls' (%) | Intact cells (%) |
|---|---|---|---|---|
| 3 | 6 | [0·5] | 18·0 | 81·5 |
| 6 | 12 | 26·5 | — | — |
| 12 | 13·5 | 42·5 | 16·7 | 40·8 |
| 24 | 20·3 | 57·7 | 15·3 | 27·0 |

From these data, the following conclusions may be drawn. The rate of breakage of yeast when the air-column is at the $A_{max}$ value, is approximately four times greater than when the column is not at $A_{max}$. Over short periods of time (6 min) the ratio of cell-walls to total broken cells remains approximately constant; if shaking is carried out for longer periods of time (e.g. 12–24 min) the cell-walls themselves begin to disintegrate. The cells are broken, and then undergo a progressive emptying of their contents, thus at an early period in the disintegration (1·5 min) 33 per cent of the cells are ruptured, and only 6·2 per cent of the nitrogen is in solution, whereas at a later period (6 min), although 65 per cent of the cells are ruptured, 22 per cent of the nitrogen is in solution. Less violent agitation, as the table shows, does not tend to empty the envelopes as completely as more violent agitation (73 per cent cells ruptured, and 20·3 per cent of the nitrogen in solution).

*Effect of weight of beads on rate of disintegration.* The effect of alterations in the rate of disintegration of yeast in the presence of various weights of ballotini beads (No. 12) was investigated by comparing the percentage of cells disintegrated in a given time, under standard conditions of air-column length $A$, amplitude of vibration and frequency. The experiments were conducted as before: the length of the air-column was adjusted to 1·85 cm below the lid ($A_{max}$), and the pot vibrated at 117 c/sec with an

amplitude of 0·3 cm. It may be seen that increasing the weight of beads from 8 g to 16 g considerably increases the effectiveness of the disintegration. An increase to 32 g of beads does not increase the disintegration further (Table VI).

Table VI. Effect of weight of ballotini beads on the disintegration of yeast. Length of air-column, 1·85 cm ($A_{max}$), amplitude 0·3 cm, frequency 117 c/sec

| Weight of ballotini beads (No. 12) (g) | Time (min) | Nitrogen in solution (%) | Cells disappeared (%) | 'Cell-walls' (%) | Intact cells (%) |
|---|---|---|---|---|---|
| 8 | 5·7 | 28·2 | 2·5 | 13·6 | 83·9 |
| 16 | 5·0 | 5·0 | 36·5 | 44·5 | 19 |
| 32 | 4·8 | 4·8 | 46·6 | 36 | 17·4 |

*Effect of* 0·25 M *sucrose on the disintegration.* The isolation of osmotically sensitive structures from micro-organisms involves disintegrating them in the presence of a stabilising material, such as sucrose, which is often used in the isolation of mitochondria from animal tissues. It is desirable that the machine should be capable of disintegrating micro-organisms under these conditions, to enable possibly sensitive structures to be isolated.

The experiments were performed as previously described except that the 1:6 yeast suspension was prepared in 0·25 M sucrose/phosphate buffer pH 7. Thirty-two g of No. 12 ballotini beads were added and the suspension vibrated in pot II for 1·3 min at 0·3 cm amplitude and 117 c/sec. At the end of this time, 39 per cent of the cells had completely disappeared, 11 per cent remained intact, and 49 per cent were in the form of cell-walls, containing various amounts of cytoplasmic material. Thirty-seven per cent of the nitrogen was in solution. The temperature of the suspension during disintegration rose from 2°C to 37°C, although the temperature rise was not responsible for the disintegration. The fractions obtained were not examined for enzyme activity. The rate of disruption is faster than in phosphate buffer alone, and this is attributed to the lesser amount of foaming which takes place, the $A_{max}$ value thus being maintained.

*Disintegration of* B. megatherium. *B. megatherium* was grown

as described. A 1:4 suspension of the organisms in 0·07 M phosphate buffer ($9 \times 10^9$ organisms/ml) was prepared and treated in pot I (at the $A_{max}$ value) with 50 g of No. 12 ballotini beads. The amplitude used was 0·3 cm and the frequency 117 c/sec. The detailed conduct of the experiments was as before, and the results are summarised in Table VII.

Table VII. Disintegration of *B. megatherium* in *Sonomec*. Amplitude 0·3 cm; frequency 117 c/sec. Length of air-column, 1·85 cm ($A_{max}$)

| Time (min) | Nitrogen in solution (%) | Cells disappeared (%) | 'Cell-walls' (%) | Intact cells (%) |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 100 |
| 2·8 | 21 | 16 | 35 | 49 |
| 5·6 | 35 | 40 | 33 | 27 |
| 11·2 | 52 | 60 | 26 | 14 |

It will be seen from this table that the disintegration of *B. megatherium* follows the same course as yeast, with the proportion of cell-walls decreasing with time and the gradual emptying of ruptured cells, as evidenced by a decrease in the ratio of broken cells to nitrogen in solution.

### *Enzyme activity of the preparations*

*Yeast preparations.* Preparations of disintegrated fresh baker's yeast were made by vibrating a 1:6 suspension of the organisms ($2{\cdot}5 \times 10^9$ organisms/ml) in pot I of the *Sonomec*, with 50 g of No. 12 ballotini beads for 5 min. A frequency of 117 c/sec and an amplitude of 0·3 cm were used. A further preparation, for comparative purposes, was made by disintegrating 50 ml of the same suspension in the Hughes press at $-23$°C. Table VIII summarises the results obtained with these two procedures. The table shows that the *Sonomec* is relatively inefficient compared with the Hughes press, in the disintegration of yeast. The proportion of cell-walls formed compared with the total cells broken is much greater with the *Sonomec*, however, than with the press. For instance (from Table VIII), although 87·8 per cent of

Table VIII. Disintegration of yeast in the *Sonomec* and in the Hughes press; details are in the text

| Method | Nitrogen in solution (%) | Cells disappeared (%) | 'Cell-walls' (%) | Intact cells (%) |
|---|---|---|---|---|
| Hughes press | 71·5 | 56·4 | 31·4 | 12·2 |
| *Sonomec* | 20 | 16 | 37·0 | 47 |

the organisms have been disintegrated, only 31·4 per cent of them are as cell-walls, whereas with the *Sonomec* 56 per cent of the cells have been disintegrated, and 36·6 per cent of them are as cell-walls.

Both preparations were fractionated centrifugally at 2°C. Whole cells were spun off at 10,000*g* for 1 min, and the resulting cell-free preparation ($S_1$) fractionated into residue ($R_2$) and supernatant ($S_2$) by centrifuging at 12,000*g* for 6 min. This supernatant was further fractionated by centrifuging for 10 min at 50,000*g* when a small pellet appeared ($R_3$), leaving a clear straw-coloured liquid ($S_3$) above. The residues $R_2$ and $R_3$ were suspended in phosphate buffer and the activity assayed as described for the succinic, lactic and alcohol dehydrogenases, and fumarase. The results are summarised in Table IX, from which it appears that the Hughes press preparations contain more lactic and succinic dehydrogenase activity than the *Sonomec* preparations, but less fumarase. Alcohol dehydrogenase activities are similar. Succinic dehydrogenase activity is restricted to particulate fractions $R_3$, the activity in $S_3$ being low. Lactic dehydrogenase does not appear to be localised in any fraction; alcohol dehydrogenase appears largely in the soluble fraction.

Corynebacterium xerosis *preparations.* A 1:4 suspension of *C. xerosis* grown on agar medium, was prepared by shaking the organisms with 10 g of No. 12 ballotini beads for 6 min in pot III, at 117 c/sec, and with an amplitude of 0·3 cm. It was not possible to make total counts of the organism due to their small size and tendency to clump, and breakage could only be estimated from the nitrogen in solution.

The original cell suspension contained 5·53 mg N/ml, and the 6,000*g* supernatant of the disintegrated organisms contained

Table IX. Enzyme activities of fractions prepared by disintegration of baker's yeast in the *Sonomec* and in the Hughes press

| Method | Fraction | Fumarase (μmoles fumaric acid /mg N/min) | Lactic dehydrogenase (μmoles dye reduced/mg N /min) | Succinic dehydrogenase (μmoles dye reduced/mg N /min) | Alcohol dehydrogenase (μmoles DPNH formed/mg N /min) |
|---|---|---|---|---|---|
| *Sonomec* | $S_1$ | 790 | 27·7 | 2·0 | 3·4 |
| *Sonomec* | $S_2$ | 340 | 36·0 | 6·3 | 3·5 |
| *Sonomec* | $S_3$ | 304 | 36·0 | 1·9 | 4·9 |
| *Sonomec* | $R_2$ | 193 | 12·0 | 3·8 | 0·5 |
| *Sonomec* | $R_3$ | 2110 | 4·8 | 8·2 | 1·9 |
| Hughes press | $S_1$ | 297 | 18·75 | 1·2 | 3·5 |
| Hughes press | $S_2$ | 416 | 19·5 | 4·3 | 2·8 |
| Hughes press | $S_3$ | 403 | 24·0 | 2·1 | 2·6 |
| Hughes press | $R_2$ | 0 | 35·0 | 22·0 | 0·2 |
| Hughes press | $R_3$ | 258 | 41·0 | 41·0 | 1·3 |

2·61 mg N/ml, i.e. 47·2 per cent of the total nitrogen was in solution. This figure indicates a high percentage rupture of the cells. Residual whole cells were removed by centrifugation at 12,000*g* for 3 min. The resulting supernatant ($S_1$) was further centrifuged at 20,000*g* for 10 min, when a pale brown residue was separated ($R_2$), leaving a straw-coloured liquid above ($S_2$).

Fifty ml of the same *C. xerosis* suspension (5·53 mg N/ml) were crushed in the Hughes press at $-25°$C. The 6,000*g* supernatant contained 2·62 mg N/ml, i.e. 47·3 per cent of the nitrogen was in solution. It was not possible in this case to separate the intact cells from the membranous (cell-wall) fraction centrifugally, and only the 12,000*g* supernatant could be assayed. The results of these assays are shown in Table X.

B. megatherium *preparations*. A 1:4 suspension of the organisms ($9{\cdot}25 \times 10^9$ organisms/ml), grown as described, was prepared in 0·07 M phosphate buffer. The suspension was shaken with 50 g No. 12 ballotini beads, in pot I of the *Sonomec*. The frequency was 117 c/sec and an amplitude of 0·3 cm for a period of 11·2 min. The original cell suspension contained 4·19 mg N/ml,

Table X. Enzyme activities of fractions prepared by the disintegration of *C. xerosis* in the *Sonomec* and in the Hughes press

| Method | Fraction | Fumarase (μmoles fumaric acid /mg N/min) | Lactic dehydrogenase (μmoles dye reduced/mg N /min) | Succinic dehydrogenase (μmoles dye reduced/mg N /min) |
|---|---|---|---|---|
| *Sonomec* | $S_1$ | 1850 | 0·3 | 4·9 |
| *Sonomec* | $S_2$ | 2030 | 0·8 | 10·8 |
| *Sonomec* | $R_2$ | 1395 | 1·85 | 13·5 |
| Hughes press | $S$ | 4180 | 4·3 | 15·8 |

and the 6,000*g* supernatant, 2·40 mg N/ml, i.e. 52 per cent of the nitrogen was in solution. A suitable dilution (1 : 250) of the organisms was taken for total counts, and differential counts on the electron microscope were performed. These data are summarised below (Table XI) together with those of a Hughes press preparation of the same suspension.

Table XI. Disintegration of *B. megatherium* in the *Sonomec* and in the Hughes press

| Method | Nitrogen in solution (%) | Cells disappeared (%) | 'Cell-walls' (%) | Intact cells (%) |
|---|---|---|---|---|
| *Sonomec* | 51·5 | 60·0 | 26·0 | 14·0 |
| Hughes press | 69·0 | 79·0 | 15·6 | 5·4 |

The crude preparations were fractionated by centrifugation at 18,000*g* when whole cells were removed. The supernatant was centrifuged at 24,000*g* for 20 min, and the residue ($R_2$) (consisting of empty membranes) and the supernatants ($S_2$) (a clear straw-coloured liquid) were assayed for enzymes. The results of the assays are summarised in Table XII. Similar fractions were obtained from *Sonomec* and Hughes press preparations. It will be seen from Table XII that the level of enzyme activity under these conditions is lower than that of similar material from *C. xerosis*, and baker's yeast. In contrast to yeast, however, there does not appear to be any localisation of succinic dehydrogenase activity in the particulate fractions. Lactic dehydrogenase is

evenly distributed, but no fumarase activity could be demonstrated in the *Sonomec* preparations, and only low activity in the Hughes press preparations.

Table XII. Enzyme activities of fractions prepared by the disintegration of *B. megatherium* in the *Sonomec* and in the Hughes press

| Method | Fraction | Fumarase (μmoles fumaric acid /mg N/min) | Lactic dehydrogenase (μmoles dye reduced/mg N /min) | Succinic dehydrogenase (μmoles dye reduced/mg N /min) |
|---|---|---|---|---|
| *Sonomec* | $S_1$ | 0 | 0·8 | 2·6 |
| *Sonomec* | $S_2$ | 0 | 0·4 | 2·3 |
| *Sonomec* | $R_2$ | 0 | 0·9 | 2·8 |
| Hughes press | $S_1$ | 1120 | 0·4 | 1·8 |
| Hughes press | $S_2$ | 0 | 0·1 | 1·9 |
| Hughes press | $R_2$ | 725 | 0 | 1·0 |

## Discussion

The rapid disintegration of micro-organisms by shaking them with small glass beads has been shown in this work to be related to the acoustic heating effect observed: lengths of air-column $A$ which are not of the critical length ($A_{max}$) cause less heat to be generated and fewer micro-organisms to be disrupted. It is considered that the mechanism of rupture in this case is similar to that in the Mickle and Nossal shakers, and to the wet-ball mills. At the $A_{max}$ length of the air-column the rate of disruption and acoustic heating is considerably increased. Why the acoustic heating should rise to a maximum is not clear; theories based on a 'liquid piston' type of motion have proved quantitatively unsuccessful in predicting the $A_{max}$ length, probably due to choice of too simple a physical model (Keller, private communication). A more likely explanation is based on the passage of shock-waves along the length of the liquid. This phenomenon is known to occur in both gases and liquids (Keller, 1953, 1954; Hudson, 1955, 1956). When using the *Sonomec* the effect is audible as a series of bursts of sound, accompanied by an absorption of energy, as indicated by an increase in current consumption by the electric motor. Pressure changes have been observed by connecting a

pressure sensitive capsule to the inside of the pot and observing these changes oscillographically. The shock-waves themselves have no disintegrating action in the absence of beads, and it is assumed that the added effectiveness of the shock-waves plus beads system is due to an increase in the velocity of the beads. How the moving beads effect the disintegration is unknown: the nature and magnitude of the forces surrounding a particle moving at high speed through a liquid are relatively unknown and the system is hydrodynamically complex.

The use of the shaker results in the production of relatively pure cell-walls. The Hughes press produces a cell-wall fraction containing much cytoplasmic material, which can be removed by shaking with glass beads in the *Sonomec* (Hunt, Rodgers and Hughes (1959) ). Cell-walls of *Lactobacillus casei*, *Corynebacterium xerosis* and *Staphylococcus albus* have been prepared.

The extracts which result from disruption in this shaker do not have the highly viscous nature characteristic of the Hughes press preparations, and they do not require the DNA-ase treatment which Hughes press extracts often need before centrifugal fractionation can be undertaken. This is advantageous in the extraction and purification of some enzymes and particulate fractions.

The *Sonomec* is also capable of disintegrating micro-organisms suspended in sucrose solutions. This is essential for the isolation of osmotically sensitive components, which freezing and thawing in the Hughes press would possibly injure.

The data for enzyme activities indicate that no advantage was gained from using the *Sonomec* over the Hughes press, and in general 'crushed' preparations exhibited higher enzymatic activities than 'Sonomec'ed' ones.

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# Continuous Centrifugation in Virus Processing

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*Summary.* Ambler's $Q/\Sigma$ relationship between the throughput of a centrifuge and its mechanical characteristics has been translated from batch data in order to predict operating conditions for continuous centrifugation in virus processing. A Sharples Laboratory Supercentrifuge was converted from batch to continuous operation and a slurry feed device was developed. A correlation between operating conditions for continuous centrifugation and amount of test virus recovered from the feed showed that the $Q/\Sigma$ values at which high concentrations of the virus were recovered were almost identical to the $Q/\Sigma$ values for significant virus recovery in batch centrifugation. Titration and gravimetric determinations of the sediments recovered from continuous centrifugation operations indicate that product purity might be influenced by operating conditions.

Operating conditions for the continuous feed of virus suspensions to a centrifuge have been translated from batch sedimentation data and used for the differential centrifugation operation in virus processing.

Differential centrifugation is a technique for the purification and concentration of viruses for possible application to the production of vaccines or other antigens. Viruses for vaccine production might be produced by inoculating chicken eggs containing living embryos with a suspension of the virus to be grown. During the period that the egg is incubated, the virus propagates within the living tissue of its host. Portions of the egg that are rich in virus are then removed from the other components of the egg and milled to a small particle size in order to release the viruses. Prior to further processing, purification of the mixture may be desired. An initial slow speed centrifugation will, in a short period of time, remove most of the extraneous protein without removing the viruses. Subsequent centrifugation at a much higher gravitational force will separate the viruses from the liquid.

Using Ambler's[1] $Q/\Sigma$ correlation between the throughput of a batch centrifuge and its mechanical characteristics, Patrick and Freeman[3] reported that there was a well defined $Q/\Sigma$ value at which significant concentrations of a test virus were sedimented from egg slurry in a batch centrifuge. $Q/\Sigma$ values were used to estimate the centrifugal force required in a larger centrifuge in order to produce the same degree of virus sedimentation as that achieved in a laboratory centrifuge.

It then became apparent that it would be advantageous to translate the batch centrifugation data to continuous operation, keeping in mind that both centrifuges must be of similar geometric proportions for the $Q/\Sigma$ relationship to be valid. The laboratory model Sharples Supercentrifuge was modified by replacing the batch bowl with a continuous discharge bowl of the same size. Centrifuge traps were provided to direct the supernatant fluid discharging from the perforated top of the bowl toward the overflow collection vessel. A copper cooling coil was installed in the air space between the bowl and the frame. During operation, Varsol* at a temperature of $-65°C$ was circulated through the coil in order to maintain the bowl temperature between 5° and 15°C. A thermocouple was placed close to the outside of the bowl in order to estimate the temperature of the material in the bowl. The reading on a thermometer, used to check the temperature of the sediment before it was removed from the bowl, showed close agreement with the thermocouple reading.

The device shown in Fig. 1 was developed to feed the slurry into the bowl at a controlled rate. Flow rates were calibrated for each size needle used by counting the drops in the flow meter and measuring the volumes at various time intervals. Calibration curves for the needles used are shown in Fig. 2. The height of the liquid level in the meter was maintained at a constant level by adjusting the air pressure to the slurry flask. The elevation of the flow meter above the centrifuge jet created enough liquid head to pass the slurry through the jet and past the fins in the bottom of the bowl. This system proved to be of satisfactory accuracy for metering very small flow rates of slurry to the centrifuge. A search of the literature revealed that only Stanley,[5] who was

* Esso Standard Oil Co., New York, N.Y.

working on the concentration of influenza viruses, approached centrifuge feed rates as low as those reached in this study.

Laboratory capacity was rather small so several lots of slurry had to be pooled to provide enough material for the tests. The pool was divided into 750 ml aliquots which were placed in one-litre flasks. One flask of slurry constituted the centrifuge feed

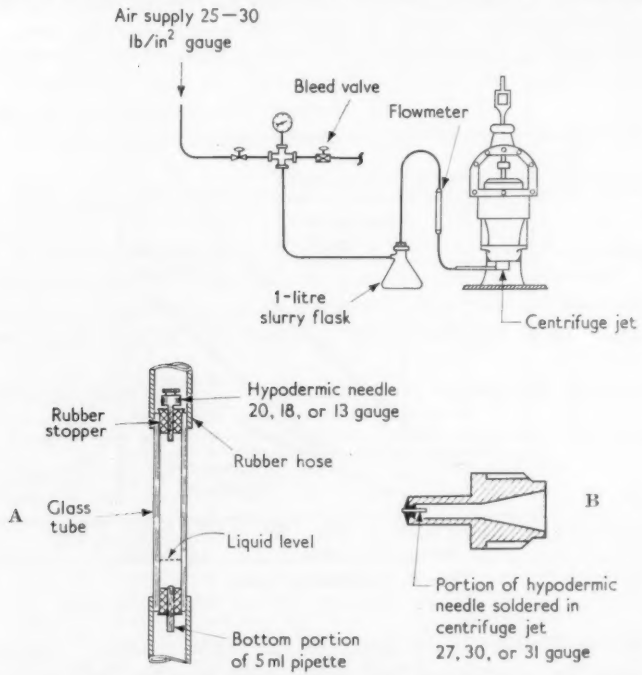


Fig. 1. Slurry feed and metering device. (Detail A: flowmeter Detail B: centrifuge jet).

for each test. Prior to operating, a small quantity of material was withdrawn from the flask for laboratory assay. Virus titre was determined by inoculating groups of mice intracerebrally with different dilutions of the virus. After observing the number of deaths in each group of test animals, the concentration of virus was calculated by the method of Reed and Muench.[4] Percentage

dry solids in the feed was determined by the method of Flosdorf and Webster.[2] The average particle size of the test virus was approximately 60 mμ.

In order to conserve slurry, the centrifuge bowl was brought up to the desired test speed and filled with 250 ml of physiological saline solution before admitting the feed. Preliminary tests indicated that supernatant fluid collected during the first 5 to 10 min of operation was lower in virus titre and percentage solids than the fluid recovered during the remainder of the run. This

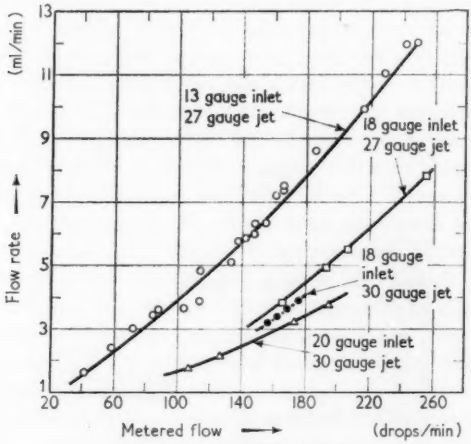


Fig. 2. Calibration curves

was due to dilution of the slurry by the saline solution. Therefore, only the supernatant fluid which came from the bowl during the final 6 min of centrifuge operation was assayed for virus titre and percentage solids. Although the volume of the supernatant fluid was measured, a material balance and virus accountability calculations could not be made. From 20 to 50 per cent of the supernatant fluid was lost during operation. This loss is believed to be attributable to the aerosolization of the supernatant fluid past the centrifuge traps.

At the completion of each run, the sediment was removed from

the bowl and weighed. The volume of slurry remaining in the bowl (bowl fluid) was also measured. Samples of the sediment were assayed for virus titre and percentage of dry solids by the methods previously cited. The initial dilution of the sediment for virus assay was prepared by milling the mixture in a Microblendor* for 2 min. This was done to break up virus agglomerates and produce consistent assays.

The volumetric measurements of the sedimented slurry under the experimental conditions of centrifugation are given in Table I.

The percentages of virus recovered from the feeds at various operating conditions are shown in Table II.

Virus recovery calculations were made by using the formula:

$$\text{Percentage recovered} = \frac{(\text{Feed titre} - \text{Supernatant fluid}) \times 100}{\text{Feed titre}}$$

It is noted in examining Table I that the weight of sediment recovered tends to decrease as the flow rate is increased. This might be expected since an increase in flow rate will decrease the retention time of the slurry in the centrifugal field. Likewise,

Table I. Bowl fluid (ml), supernate (ml) and sediment (g) recovered after continuous centrifugation[a]

| Flow rate ml/min | Bowl rotation (rev/min × 1,000) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 15 | | | 25 | | | 40 | | | 50 | | | 54 | | |
| | B.F. | Sup. | Sed. | B.F. | Sup. | Sed. | B.F. | Sup. | Sed. | B.F. | Sup. | Sed. | B.F. | Sup. | Sed. |
| 4·0 | * | * | * | * | * | * | 130 | 300 | 60 | * | * | * | 190 | 110 | 39 |
| 5·0 | 160 | 642 | 62 | 160 | 660 | 63 | 168 | 174 | 62 | * | * | * | * | * | * |
| 6·0 | * | * | * | * | * | * | * | * | * | 164 | 320 | 58 | * | * | * |
| 7·6 | * | * | * | * | * | * | * | * | * | * | * | * | 164 | 388 | 58 |
| 25·0 | 158 | 686 | 62 | 172 | 640 | 54 | 202 | 622 | 49 | * | * | * | * | * | * |
| 50·0 | * | * | * | 176 | 556 | 49 | 200 | 530 | 49 | * | * | * | * | * | * |

[a] Measurements based on slurry feed of 750 ml plus 250 ml of saline.
* Not investigated.

Table II shows that an increase in flow rate results in a smaller percentage of virus recovered from the feed. But, this table also indicates that an increase in bowl speed at a constant flow rate

* Waring Products Corp., New York, N.Y.

Table II. Percentage of virus recovered from feed

| Flow rate ml/min | Bowl rotation (rev/min × 1,000) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 15 | 25 | 30 | 40 (%) | 50 | 52 | 54 |
| 2·1 | * | * | * | * | * | 100·0 | * |
| 4·0 | 84·0 | * | * | 84·0 | * | * | 99·2 |
| 5·0 | 56·0 | 84·5 | * | 73·0 | * | * | * |
| 6·0 | * | * | * | * | 99·0 | * | * |
| 7·6 | * | * | * | * | * | * | 91·0 |
| 25·0 | 43·5 | 0·0 | * | 83·0 | * | * | * |
| 35·0 | * | * | 63·4 | * | * | * | * |
| 50·0 | * | 29·0 | * | 42·0 | * | * | * |

* Not investigated.

results in an increase in percentage of virus recovered from the feed. The seeming paradox of sediment weight decreasing with increases of bowl speed at constant flow rates while virus recovery increases under the same conditions might be explained as the effect of the sediment compacting more at higher bowl speeds than the recovery increasing. This would give a net decrease in the wet weight of sediment even though the dry weight, which is proportional to the actual virus titre, is increasing.

It is also possible that the smaller quantities of sediment recovered at increased bowl speeds are more highly purified than the sediments recovered from operations at smaller centrifugal forces. This could happen if there is a significant difference in specific gravities between the pure viruses and the remaining impurities in the feed. It would be rather presumptious to believe that the initial low speed centrifugation (2,000 rev/min in an International Centrifuge, Model PR–1), to which the original slurry is subjected in the differential centrifugation process, will rid it of all extraneous proteinaceous solids.

Table III shows the percentage of total dry solids in the supernatant fluid and the percentage of total dry solids removed from the feed at various operating conditions.

The percentages of total dry solids removed from the feed were calculated from the quantities of total dry solids that were found empirically in the feed and supernatant fluid. Feed solids ranged between 6·5 and 8·6 per cent. It might be expected from referring

Table III. Percentages of total dry solids in supernate (Sup.) and total dry solids removed from feed (F.)

| Flow rate ml/min | Bowl rotation (rev/min × 1,000) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 15 | | 25 | | 40 | | 50 | | 54 | |
| | Sup. | F. | Sup. | F. | Sup. | F. | Sup. | F. | Sup. | F. |
| | % | | | | | | | | | |
| 4·0 | * | * | * | * | 5·6 | 33·0 | * | * | * | * |
| 5·0 | 5·8 | 20·7 | 4·7 | 36·7 | 3·9 | 41·0 | * | * | * | * |
| 6·0 | * | * | * | * | * | * | 5·4 | 35·0 | * | * |
| 7·6 | * | * | * | * | * | * | * | * | 6·5 | 24·0 |
| 25·0 | 4·6 | 29·6 | 5·3 | 29·3 | 5·9 | 28·5 | * | * | * | * |
| 30·0 | * | * | 4·9 | 30·0 | 6·2 | 27·0 | * | * | * | * |

* Not investigated.

to Table I again that a decrease in sediment weights would be reflected by an increase in supernatant fluid solids in Table III. Unfortunately, the two tables cannot be correlated because the weights shown in Table I are wet weights.

Values of $Q/\Sigma$ were then calculated for the various conditions of centrifugation. The formula used for calculating $\Sigma$ values for the Sharples Laboratory Model Supercentrifuge for continuous operation is:

$$\Sigma = \frac{2\pi l\omega^2}{g}\left(\frac{3}{4}r_2{}^2 + \frac{1}{4}r_1{}^2\right)$$

where, $g = 981$ cm/sec$^2$, $r_2 = 2{\cdot}21$ cm (radius of outer surface of liquid layer), $r_1 = 1{\cdot}07$ cm (radius of inner surface of liquid layer), $l = 19{\cdot}7$ cm (length of bowl), and $\omega$ = angular velocity about the axis of rotation, radians per sec.

Table IV shows the calculated values of $Q/\Sigma$ and the percentage of virus recovered from the feed for various conditions of centrifugation.

The calculated $Q/\Sigma$ values were plotted against the percentage of virus recovered from the feed to the centrifuge (Fig. 3). The plot indicates graphically that there is a break point in the curve at about 0·5 to $1{\cdot}5 \times 10^{-8}$ cm/sec where there is no significant increase in virus recovery for a decrease in $Q/\Sigma$. Although there is some virus recovery from the sediment at every condition of centrifugation as shown in Table II, significant concentrations of

Table IV. Summary of virus recovery from feed and $Q/\Sigma$ values for continuous operation of the Sharples Laboratory Centrifuge

| Centrifuge, rev/min × 1,000 | Flow rate ml/min. | Virus recovered from feed, % | $Q/\Sigma$ values, (cm/sec) × $10^{-8}$ |
|---|---|---|---|
| 15 | 4·0 | 84·0 | 5·420 |
| 15 | 5·0 | 56·0 | 6·770 |
| 15 | 25·0 | 43·5 | 33·850 |
| 25 | 5·0 | 84·5 | 2·441 |
| 25 | 25·0 | 0·0 | 12·205 |
| 25 | 50·0 | 29·0 | 24·410 |
| 30 | 35·0 | 63·4 | 11·890 |
| 40 | 4·0 | 84·0 | 0·765 |
| 40 | 5·0 | 73·0 | 0·957 |
| 40 | 25·0 | 83·0 | 4·780 |
| 40 | 50·0 | 42·0 | 9·560 |
| 50 | 6·0 | 99·0 | 0·734 |
| 52 | 2·1 | 100·0 | 0·237 |
| 54 | 4·0 | 99·2 | 0·419 |
| 54 | 7·6 | 91·0 | 0·795 |

virus (90 per cent or more) are not removed from the feed until a $Q/\Sigma$ value of 0·5 to $1{\cdot}5 \times 10^{-8}$ cm/sec is reached. This result is almost identical to the $Q/\Sigma$ values calculated for batch centrifugation operations.[3]

The break point at centrifugation conditions where significant quantities of virus are sedimented is again illustrated by plotting virus titre in the sediment and in the supernatant fluid against $Q/\Sigma$ (Fig. 4). It can be seen that severe conditions of centrifugation do not harm the virus in that the concentration of virus in the sediment increases as the concentration of virus in the supernatant fluid decreases. Since the concentration of virus is expressed as the negative logarithm to the base 10 of the lethal dose for 50 per cent of the animals challenged, there is a large difference in concentration between the virus in the sediment and in the supernatant fluid. Even though the titre of the supernatant fluid is high, it is at least 50 per cent lower than the titre of the sediment. At the lower $Q/\Sigma$ values, the titre of the supernatant fluid may be only 1/10,000 the titre of the sediment.

The results of this study have shown that it is possible, by using Ambler's $Q/\Sigma$ relationship, to translate batch centrifuge operating

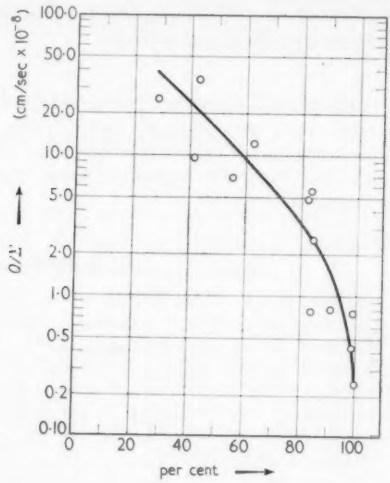


Fig. 3. $Q/\Sigma$ vs. percentage of virus recovered from feed

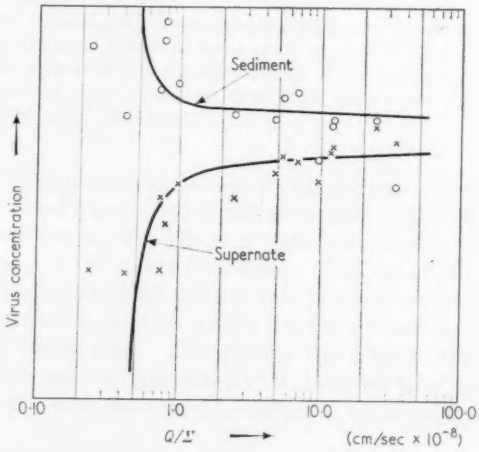


Fig. 4. Concentration of virus in sediment and supernate vs. $Q/\Sigma$

conditions to conditions for continuous operation of the Sharples Laboratory Supercentrifuge.

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# Effect of Antifoam Agents on Oxygen Transfer in Deep Tank Fermentations*

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*Summary.* Development of a foam breaker which operates on the principle of rapid acceleration of foam through a nozzle has allowed large-scale aerobic fermentations to be run without addition of antifoam agents. Fermentations have been investigated over a wide range of aeration levels in which oxygen supplied to the organisms was the limiting growth factor.

With *Torulopsis utilis* grown in a 5 per cent molasses medium, oxygen utilization followed an exponential pattern during the early phase of growth, with carbon dioxide production equal to the consumption of oxygen. At the point where oxygen supply to the organism became limiting, anaerobic fermentation began, and the consumption of oxygen remained constant. The level of oxygen absorption beyond which the respiratory quotient exceeded unity was used as a measure of limiting oxygen supply.

Addition of three types of antifoam compounds to *T. utilis* fermentations resulted in a marked depression of oxygen consumption, and a decrease in cell production. Incremental addition of small quantities of antifoam to maintain a constant foam level in the fermentor depressed the oxygen absorption rate to a lesser extent. Sulphite oxidation rates did not reflect the true value of oxygen absorption rates found for fermentations without addition of antifoam.

Chemical antifoam agents are normally used to control foaming in submerged aerobic fermentations. Attempts have been made to determine the effect of these compounds on oxygen absorption rates (OAR) in the fermentor. Corman *et al.*[4] used three different types of antifoam compounds in catalyzed sulphite solutions, and reported that OAR values were depressed to about 12 per cent of the initial rate. Diendoerfer and Gaden[5] added 0·01 per cent of a solution of Alkaterge C in lard oil to a filtered fermentation broth, and measured a decrease of almost 50 per cent in the

* Issued as N.R.C. No. 5607.

oxygen absorption coefficient. Pfeifer *et al.*[8] suggested that excessive amounts of 0·75 per cent octadecanol in ethanol reduced the fermentation rate and decreased the yield in an *Aspergillus terreus* fermentation, though no quantitative data were given.

Chain and Gualandi,[2] using a vortex type fermentor containing a culture medium, reported a ten-fold increase in OAR when 0·02 per cent of an antifoam agent was added. This increase was explained as resulting from the breakdown, by the antifoam agent, of a stable recirculating emulsion of oxygen-depleted air bubbles, allowing fresh air to be drawn into the vortex. This would also explain increases in OAR reported after addition of antifoam agents in Waldhof-type fermentors[1] and in shake flasks.[9]

In this study the objective was to obtain a direct measure of the effects of chemical antifoam agents on OAR values in submerged aerated fermentation. This required: (a) that comparable fermentations be run both with and without the addition of antifoam agents, and (b) that oxygen supply to the organisms be the limiting growth factor. The first requirement was met by developing a mechanical foam breaker* which permitted controls to be run at high aeration rates, and the second by using a complete nutrient medium based on molasses. An initial series of fermentations contained no antifoam agents while 0·1 per cent of one of the common antifoams was added in succeeding series. Measurements of OAR obtained by determination of oxygen in the effluent air from the fermentor showed that antifoams had a marked depressing effect on oxygen transfer.

### *Equipment*

A 200-gal (U.S.) stainless steel fermentor of fully baffled design was used in this investigation. The fermentor had a diameter of 3 ft and was fully jacketed about the area in contact with the nominal working capacity of 120 gal. A 13-in. impeller, having 8 vertical vanes (2·5 in. × 3·25 in.) attached to a 9-in. disc, was positioned approximately at one-third of the depth of the liquid from the bottom. The impeller was driven by a 5 h.p. two-speed motor operating through a geared speed reducer. Interchangeable

* Patent pending—Canadian Patents and Development Ltd; National Research Building, Ottawa, Ontario, Canada.

gears provided seven speeds ranging from 100–280 rev/min at low input speed, and 200–560 rev/min at high speed. A mechanical seal with pressure lubricator was installed to prevent leakage around the impeller shaft.

A metered flow of filtered air was centrally directed to the underside of the impeller by a single hole sparger. Effluent gas and foam passed from the fermentor through the foam breaker nozzle (Fig. 1) at velocities of 100–300 ft/sec. This sudden acceleration resulted in almost complete destruction of the foam.

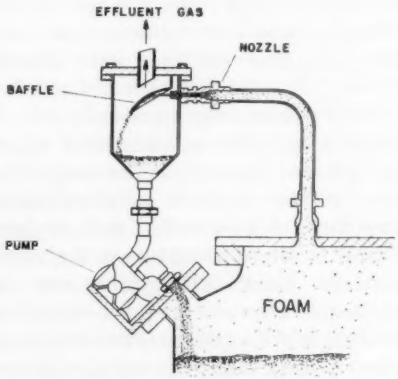


Fig. 1. Foam breaker in operation

Liquid from the broken foam was deflected downward by an inclined concave baffle and returned to the fermentor by a positive displacement pump. The baffle was electrically insulated from the body of the foam breaker. Liquid issuing from the nozzle against the baffle completed an electrical circuit, and permitted the pump to operate only when the fermentation was actively foaming. Effluent gas escaped through the outlet pipe leading from the top of the foam breaker. During active foaming periods, pressure in the fermentor seldom exceeded 5 lb/in$^2$ gauge. Interchangeable nozzles of varying internal diameter were used to impart sufficient velocity to the foam, depending on the rate of air flow through the fermentor.

### *Fermentation and Analytical Methods*

*Torulopsis utilis*, NRRL Y900 was maintained on slants of medium containing, per litre, 3 g malt extract, 3 g yeast extract, 5 g peptone, 10 g glucose and 20 g agar. Inoculum was grown in shaken cultures in two 24-h stages using a medium containing, per litre, 20 g glucose, 6 g monobasic ammonium phosphate, 0·2 g monobasic potassium phosphate, 0·25 g magnesium sulphate heptahydrate, 1 g trisodium citrate and 2·5 g L-asparagine. One per cent inoculum was used.

The fermentor was charged with 120 gal (U.S.) of medium containing, by weight, sugar beet molasses 5 per cent, urea 0·066 per cent, calcium phosphate 0·033 per cent, dibasic ammonium phosphate 0·033 per cent and concentrated sulphuric acid 0·12 per cent. The initial pH was approximately 5·5. This medium permitted exponential growth to the point at which oxygen became the limiting factor throughout the range of aeration levels studied. Samples of the medium were withdrawn from the fermentor at the time of inoculation and at hourly intervals commencing after 6 h, which constituted the normal lag phase for the fermentation. Total carbohydrate was determined by the anthrone method.[7] Per cent wet cell volume was measured using a DeLaval Gyro-test centrifuge fitted with tapered test tube assembly. Optical density and pH were also determined.

Air was supplied to the fermentor at a constant rate of 5 standard cubic feet per minute through a calibrated rotameter, and passed through a cylindrical, porous, stainless-steel filter wrapped in glass wool. Effluent gas was sampled at 30-min intervals following the lag phase. During the period of most active growth, sampling intervals were reduced to 20 min. Samples were analyzed for oxygen, carbon dioxide, and nitrogen using a standard Orsat gas analysis apparatus. To correct for oxygen depletion of air passing through the fermentor, the log-mean partial pressure was used in calculating the rate of oxygen absorption.[3]

The temperature of the fermentation was automatically controlled at $30 \pm 0{\cdot}5$°C. Pressure in the fermentor above atmospheric was recorded using a differential type pressure recorder calibrated from 0 to 200 in. of water.

Four fermentations were run at impeller speeds of 100, 125, 150 and 188 rev/min without addition of antifoam. Absolute pressures in the fermentor averaged 1·12, 1·15, 1·24 and 1·32 atm respectively. A second group of fermentations, to which 0·1 per cent of Dow Corning Antifoam A (DCA) had been added prior to sterilization, was carried out at the same impeller speeds. This concentration of antifoam was reported to give the maximum depression in OAR when added to a fermentation broth.[5] Since no foaming occurred during this series, it was necessary to throttle the valve in the effluent gas line to maintain pressures in the fermentor similar to those found in the control series.

Two fermentations were run at an impeller speed of 150 rev/min to determine the effect of 0·1 per cent Hodag K–4 and of 0·1 per cent lard oil respectively. Each of these compounds was sterilized separately and added to the fermentor at the time of inoculation.

Hodag K–4 and lard oil were added to two fermentations in increments of 15 ml whenever foam entered the foam breaker, to determine whether addition of a minimum amount of antifoam to control foaming had any effect on OAR. When this occurred, the electrical circuit was energized and the foam breaker pump would operate. Two additions of Hodag, one immediately after inoculation and the second 1·75 h later, were sufficient to prevent foam from leaving the fermentor. With lard oil, 30 ml was required immediately after inoculation, followed by two more additions of 15 ml each at 3 and 3·5 h.

### *Results*

Patterns for typical fermentations at an impeller speed of 150 rev/min are shown without addition of antifoam (Fig. 2) and with addition of 0·1 per cent DCA (Fig. 3). The pH followed a consistent pattern in all fermentations, dropping to a value of 3·5–4·0 when available carbohydrate was exhausted. This occurred 12–14 h after inoculation. Percentage wet cell volume was appreciably lower when the antifoam compound was used.

The fermentation patterns show that prior to reaching the point where oxygen supply became limiting, the respiratory quotient (RQ) was unity, the quantities of oxygen consumed and carbon dioxide produced being determined directly using the Orsat

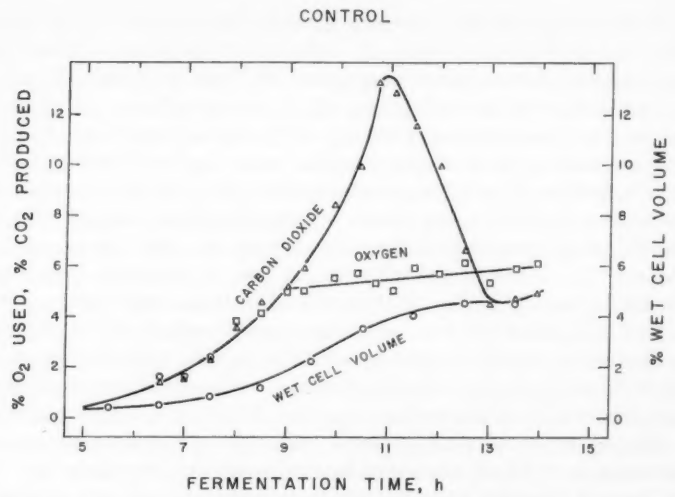


Fig. 2. Fermentation pattern for *T. utilis* without addition of antifoam. Impeller speed: 150 rev/min. Superficial air velocity: 42·5 ft/h

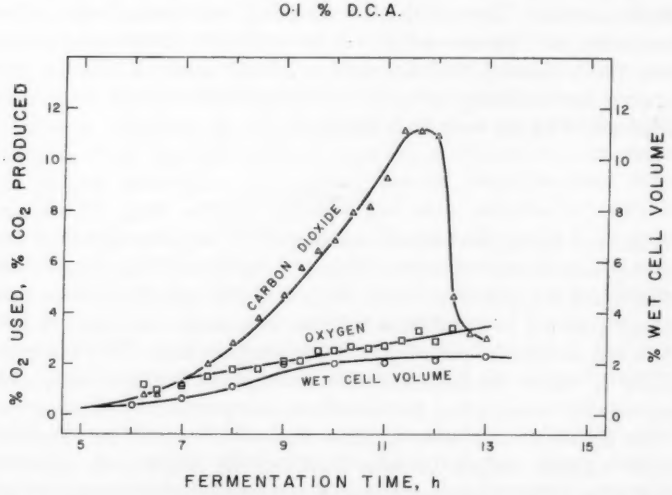


Fig. 3. Fermentation pattern for *T. utilis* with addition of 0·1 per cent DCA. Impeller speed: 150 rev/min. Superficial air velocity: 42·5 ft/h

apparatus. After oxygen became limiting, the RQ exceeded unity and the excess of carbon dioxide produced over the amount of oxygen consumed gave a depressed value for oxygen, carbon dioxide and nitrogen. These values were converted to a basis of 79 per cent nitrogen in the effluent gas to obtain the true values for oxygen and carbon dioxide.

Using the average value for the rate of oxygen utilized after oxygen supply became limiting, OAR for the series of fermentations at 100, 125, 150 and 188 rev/min, with and without addition of 0·1 per cent DCA, are shown in Table I. Values of OAR for copper-catalyzed sulphite solutions under identical air flow rates and impeller speeds are included for comparison. The sulphite oxidation rate (SOR) values are corrected to the 0·8 power of the absolute pressure, derived from the data of Elsworth *et al.*[6] so that they correspond with pressures obtained during the fermentation. The rates given in Table I were plotted on a logarithmic graph (Fig. 4) and varied as the cube of the impeller speed. This is in agreement with theoretical mixing equations.

Table I. OAR for *T. utilis* fermentations at different impeller speeds

| Impeller speed, rev/min | Oxygen absorption rate, mmole/l. h | | SOR* |
|---|---|---|---|
| | Control | 0·1% DCA | |
| 100 | 12·3 | 6·8 | 24·3 |
| 125 | 26·0 | 9·7 | 47·0 |
| 150 | 47·0 | 18·8 | 74·0 |
| 188 | 80·5 | 39·2 | 116·0 |

Superficial air velocity: 42·5 ft/h
* Sulphite oxidation rate

Addition of 0·1 per cent Hodag K–4 (Fig. 5) and lard oil (Fig. 6) reduced the OAR. Each of the antifoams tested affected the pattern of oxygen uptake in a different manner. DCA caused a sharp initial depression, followed by a steady increase in the rate of oxygen uptake during the remainder of the fermentation. Hodag K–4 exhibited a two-stage effect, a strong initial depression with OAR remaining constant, followed by a well defined transition to a higher oxygen uptake level. Lard oil showed the least effect on OAR and cell production. The relative effect of these antifoam compounds on OAR is shown in Table II.

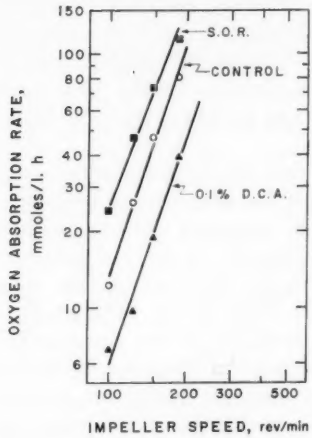


Fig. 4. Effect of impeller speed on oxygen absorption rate. Superficial air velocity: 42·5 ft/h

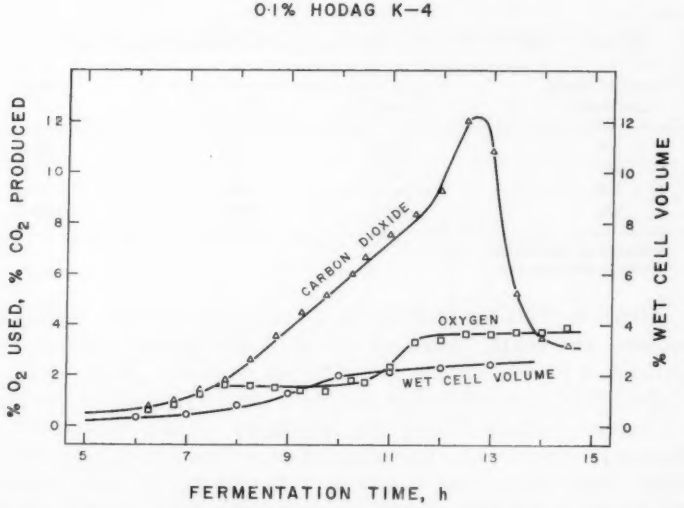


Fig. 5. Fermentation pattern for *T. utilis* with addition of 0·1 per cent Hodag K–4. Impeller speed: 150 rev/min. Superficial air velocity: 42·5 ft/h

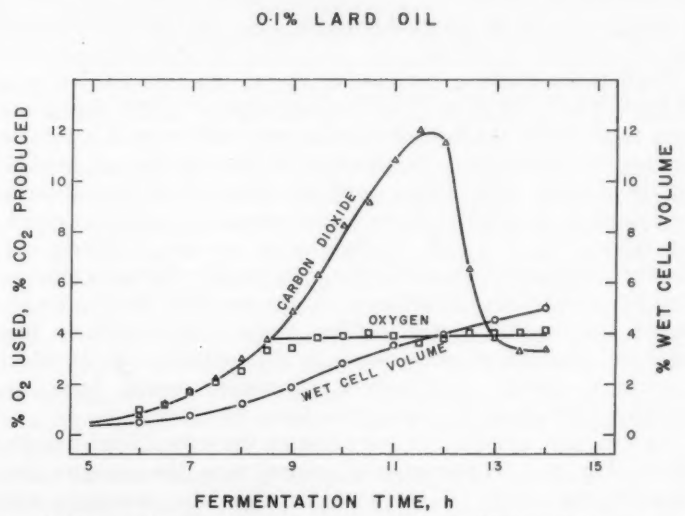


Fig. 6. Fermentation pattern for *T. utilis* with addition of 0·1 per cent lard oil. Impeller speed: 150 rev/min. Superficial air velocity: 42·5 ft/h

Table II. Effect of antifoam on OAR in *T. utilis* fermentations

| Fermentation | Oxygen absorption rate, mmole/l. h |
|---|---|
| Control (no antifoam) | 47·0 |
| DCA 0·1 per cent | 18·8 |
| Hodag K–4 0·1 per cent | 20·4 |
| Lard oil 0·1 per cent | 31·4 |

Impeller speed: 150 rev/min
Superficial air velocity: 42·5 ft/h

When foaming was controlled by adding small amounts of antifoam as required the depression in OAR was less pronounced. Only 30 ml of Hodag K–4 was required to maintain the fermentation at the point of incipient foaming. No stepwise increase in oxygen uptake rate was observed when Hodag antifoam was added in this manner. Addition of 60 ml of lard oil gave similar results. Oxygen absorption rates were 30·0 and 35·0 mmole/l. h respectively. This represents 65 and 75 per cent of the value obtained when no antifoam was used.

## Discussion

Each of the antifoam compounds caused a substantial lowering of OAR when added to *T. utilis* fermentation. This depression may result from (a) formation of larger bubbles with a smaller surface to volume ratio; (b) decrease in the time the gas bubbles are in contact with liquid medium when foam formation is restricted, or (c) additional gas–liquid interfacial resistance resulting from addition of the surface active material. Starks and Koffler[9] have shown that addition of 'penicillin defoamer' had no significant effect on the diffusion of oxygen from air into freshly boiled and rapidly cooled distilled water. It is unlikely that addition of antifoam compounds to fermentation media which contain a variety of dissolved substances would have any additional effect on gas–liquid interfacial resistance.

The oxygen uptake rate increased as the cube of the impeller speed (Fig. 4). In the series of control runs, the pressure drop through the nozzle of the foam breaker also increased with increasing impeller velocities, even though the air flow rate was constant. This effect must have been caused by increased foam density and/or stability. Such characteristics would result if the foam were composed of large numbers of small bubbles. Thus the increase in OAR with increasing impeller velocities must have resulted from the larger interfacial surface area generated by dispersing the same amount of air into a greater number of smaller bubbles.

Examination of the interior of the fermentor after each run gave an explanation for the difference in the effect of the three antifoams on OAR. The silicone base compound, DCA, formed a thick gummy ring on the walls of the fermentor and on the impeller shaft approximately 4 in. above the liquid level in the unsparged fermentor. The progressive decrease in the concentration of the antifoam in the medium as it separated on the wall of the fermentor would explain the gradual increase in OAR as the fermentation proceeded (Fig. 3). Lard oil formed a greasy layer in the same manner as the DCA but not to the same extent. When Hodag K–4 was used the entire inside surface of the fermentor became coated with the antifoam compound. During the early stage of the fermentation it is possible that

foaming was entirely suppressed by Hodag K–4, resulting in a strong suppression of OAR (Fig. 5). Following this period the foam layer rose in the fermentor until the initial free volume was completely filled. A relatively sudden increase in OAR would result from increased hold-up of air bubbles in contact with fermentation liquid in the foam. Further evidence in support of this postulate is provided from the observation that the higher oxygen uptake rate was approximately the same as the constant rate determined in the fermentation in which foaming was controlled by incremental addition of this antifoam agent.

Comparison of OAR values obtained from sulphite oxidation data, and from fermentations with and without addition of antifoam (Fig. 4) indicates that results from the sulphite method for estimating aeration requirements in an actual fermentation must be accepted with reservations. Use of a standard fermentation as herein reported with analysis of effluent air to determine the actual rate of removal of oxygen throughout the course of the fermentation will give direct and positive results for OAR. This method may prove even more important for mycelial type fermentations, where increases in concentration of the organism are known to cause extreme depressions in oxygen absorption rates.[5]

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# Theoretical Aspects of Sterilization of Tissue Culture Medium by Depth Filtration

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*Summary.* Filtration theory is briefly reviewed with regard to possible application to sterilization by filtration. Of the different devices tested, Seitz asbestos sheets, acid-treated to remove toxic materials, offered most advantages for routine use and scale-up potential. The standard law of Hermans and Bredée best expressed mathematically, the filtration of tissue culture media sterilized by Seitz No. 6 laboratory filters. Data are presented showing the correlation between the performance of the Seitz No. 6 filter, and the Seitz No. 200 plate and frame press (involving a scale-up ratio of up to 400 to 1). An expression:

$$t = 3{\cdot}25\,\Delta P^{-0{\cdot}627}/(A/V_f + 0{\cdot}001\,\Delta P - 0{\cdot}036)$$

derived from experimental data obtained, expressing time in terms of pressure, surface area, and volume of filtrate, enabled prediction of filtration parameters with accuracy acceptable for engineering purposes for unclarified media, and greatly improved for media which had been initially clarified.

The influence of streaming potential is discussed with regard to its influence on the course of a filtration.

## Introduction

Although many bacteriological media may be sterilized by heat, such is not the case for some types, particularly those within the tissue culture field. Media containing sugars, for example, are notoriously labile to heat. Those employing sera and other biological fluids are even more susceptible to denaturation or other chemical changes. In tissue culture research, the media generally cannot be processed with heat. In this presentation, the techniques and theory of sterilization of media by filtration are explored, particularly with respect to scale-up application.

According to Smith and Martin,[13] filters used for the removal of bacteria from liquids are of four main types: (1) diatomaceous earth filters (Berkefeld, Mandler), (2) unglazed porcelain filters (Pasteur–Chamberland, Doulton, Massen), (3) asbestos filters (Seitz), and (4) sintered-glass filters.

In addition to filters of these types, collodion membranes are occasionally used to separate bacteria from liquids. Commercial filters of this nature would be represented by the membrane filter.

A filtration-designed sterilization of a liquid has theoretical aspects most in common with the larger-scale industrial filtrations in which liquids are processed for removal of small amounts of suspended material.* This is particularly true if the liquid, or any of its components, has been previously clarified to any extent. Therefore the sole purpose of the operation is the removal of any micro-organisms (for example bacteria) that may be present. Naturally these would be expected to be few; if large numbers were present, fermentive changes would render the material valueless for subsequent use.

Such filtrations would also be characterized by the large initial resistance to flow afforded by the pad or device used to retain the bacteria. This resistance would increase as material accumulated both on and within the filtering medium itself. For these reasons, the behaviour characteristics of such a system would not be expected to be exactly similar to those of the majority of industrial filtrations in which the initial resistance is quite low and builds up with the accumulation of the suspended material or 'cake'. In instances such as this, the deposition of the cake is required before effective filtration can be accomplished.

## Technical Considerations

Although the theory of filtration has been treated by several authors,[4, 5, 11] no one, to the best of our knowledge, has considered the special case of filtration for the sole purpose of rendering a liquid material sterile. Most investigators have given consideration to the so-called cake filtration—a common method in the chemical industry—in which a liquid containing a relatively

* According to Grace[5] the filtration of dilute suspensions containing less than 100 ppm by volume of solids has become of great practical importance.

large amount of a solid is filtered through a cloth or other suitable material, gradually forming a filter-cake which actually is the filtering medium. Carman[2] was probably the first to give a complete and correct mathematical explanation of the theory of this type of filtration in terms of the experimental data.

Hermans and Bredée[7,8] arrived independently at a similar mathematical relationship. They point out that, although Ruth, Montillon and Montonna[11] were the first to establish clearly the importance of Poiseuille's law in cake filtration, the expression for the relationship between the volume filtered $V$, and time $t$, having the semi-empirical form

$$(V+c)^2 = k(t+t')$$

where $c$ and $t'$ are constants, does not always adequately express this type of filtration.

Hermans and Bredée[8] point out that in industrial research, many other types of filtration are encountered (such as filtration of viscose, or clarification of beer) which frequently follow other laws. To these two examples may be added the polishing of oils used in the hydraulic mechanisms of aircraft. In their earlier paper, these authors[7] were probably the first to outline the theory and mathematical treatment of these laws, showing their connection with the cake-type filtration and experimental data to support their use.

In the later paper,[8] they attempted to indicate the practical value of these expressions for the study of industrial filtrations. They separated filtration types into four categories or laws according to the theory and behaviour involved in describing the course of the filtration.

By regarding cake filtration as the flow of liquid through a group of parallel, cylindrical, capillary tubes of constant diameter and increasing length, such flow obeying Poiseuille's law for capillary flow, Hermans and Bredee[7] derived the following equation for cake filtrations:

$$kV/2 = t/V - 1/S_0$$

in which $k$ is a constant and $S_0$ is the initial flow rate.

By plotting $t/V$ against $V$, a straight line is obtained. The constants $k/2$ and $1/S_0$ can readily be determined from the slope

and intercept. The constant $k/2$ indicates the rate of clogging and $1/S_0$ the initial resistance of the filtering medium.

The behaviour of flow according to this equation will be followed by nearly all liquids with a relatively large amount of precipitate or suspended material. On the other hand, liquids containing a relatively small amount of precipitate may reasonably be expected to follow other laws in that no appreciable formation of a cake takes place. The particles to be filtered are of such a size and type that a gradual blocking of the pores of the filter material itself takes place.

Examples of this type of filtration may be found in viscous solutions of high molecular organic substances, such as solutions of cellulose derivates or starch. Similarly, very dilute suspensions of finely dispersed pigments would behave in a similar manner.

Hermans and Bredée[7] were able to derive two other filtration equations by considering this type of filtration in the following manner. If the filtering medium is regarded as a collection of parallel capillary tubes of constant diameter and length which become blocked in such a way that every time a particle passes into a tube the latter is completely sealed, then a linear relation exists between the rate of flow $S$, and the volume filtered $V$:

$$S - S_0 = kV$$

According to the authors, the subsequent relation between $V$ and $t$

$$V = S_0(1 - e^{-kt})$$

is more complicated than that in the case of the cake filtration, and the constants within the expression cannot be found easily and cannot be applied for practical purposes. They term this relationship the 'law of complete blocking'.

On the other hand, if the filter is considered to consist of a set of parallel capillary tubes of constant length, the diameter of which decreases gradually and regularly by the adsorption of precipitate against the walls of the tubes in such a way that the inside volume of the tubes decreases proportionally to the volume of filtrate passed through, a new expression may be derived:

$$kt/2 = t/V - 1/S_0$$

which is termed the 'standard law'. Most of the filtrations

encountered, according to Hermans and Bredée, which are met with on a practical scale and do not obey the cake law, follow this mode of filtration.

The fourth law proposed by these authors is the 'intermediate law', which is said to follow the characteristics of both the caking law and the standard law. Although they were not able to visualize the exact mode of plugging of the filter material, they arrived at the mathematical expression:

$$kt = 1/S - 1/S_0$$

from entirely empirical considerations.

The detailed derivation of all these equations may be found in the original manuscript.[7] The authors demonstrated that all of their derived relationships or laws could be reduced to a common differential equation which can be adapted to any type of filtration by adjusting the values of two constants. Thus for constant pressure filtration

$$\frac{d^2t}{dV^2} = k\left(\frac{dt}{dV}\right)^n$$

where the value of $n$ would define the mode of filtration occurring, with the value of $k$ dependent on the conditions of the filtration system.

Gonsalves[6] visualized an additional equation by assigning the value 1/2 to $n$, but he attached no practical importance to the law. He also demonstrated that the same equations could be derived by starting with physical 'pictures' of the method of filter clogging different from those visualized by Hermans and Bredée. He maintained that the standard law, for which $n = 3/2$, did not properly describe all viscose filtrations, and that a time-consuming calculation of the two constants would be necessary to describe the system fully.

Grace,[5] in an excellent work on the structure and performance of filter media, correlated the pore structure of a number of filtering media with their plugging rates. These plugging rates were determined from standard-law plotting of data collected during clarification of a dilute carbonyl iron suspension. Grace found that such filtrations could be divided into three periods. The initial period conformed to no filtration theory. An extended period of filtration, characterized by the standard law, then

followed until 50–80 per cent of the filtrate volume theoretically required to cause complete plugging had passed. The filtration then passed through a transition zone to conform to the cake law in the final period.

In this paper, the basic factors underlying the mechanisms of sterilization by filtration are discussed. Equations relating filtration rates to basic operating conditions were developed for a tissue culture medium from the results of small-scale laboratory filtrations. The validity of these equations was checked by correlation with a much larger filter which has been used for routine sterilizations of media in the laboratory.

## Experimental Procedures and Methods

Experience with the different types of filters, such as the Berkefeld, Selas, fritted glass, and Seitz, revealed that those other than the Seitz possessed at least three undesirable characteristics. The first is the necessity for exacting cleaning procedures after use. The second is the requirement of periodic tests with bacteria such as *Serratia marcescens*[13] to detect cracks which may have developed with consequent leaking. The third characteristic is the rapid reduction in flow after initiation of filtration. Thus the returns on the time involved in preparation of the filter, sterilization and actual use are rather low.

On the other hand, asbestos filters of the Seitz type seemed to have advantages worthy of exploitation if they could be made to serve the desired ends. These ends are relatively high rates of flow, quality control at the factory level rather than within the laboratory, and disposability.

The Seitz filter was found to possess one undesirable feature in that the initial product was occasionally found to contain asbestos fibres. By reprocessing the initial output, this disadvantage was overcome.

Hormann[9] describes a new asbestos pad designed to increase flow rates and decrease plugging rates. Such a pad provides 'depth' filtration because it has gradient porosity. Surface sealing or clogging of the pads is eliminated, resulting in higher rates of flow, fewer pad changes and economy in filtering operations.

The use of such pads in this laboratory was initially unsuccessful due to the presence of toxic substances within the pads. This feature rendered them objectionable for preparation of such types of media as that used for tissue culture. These toxic materials were found to be removable by washing with 0·1 N HCl followed by 0·1 N $NaHCO_3$ and a triple rinse with distilled $H_2O$. The manufacturer,* now treats the pads in a similar fashion.

Since use of Seitz asbestos sheets was found to be the best means for media sterilization by filtration, at least for the purposes of this laboratory, a knowledge of the basic characteristics of this filtration system was considered appropriate to any tissue culture research in which large volumes of sterile media might be required. Consequently, an investigation to obtain data on laboratory filtration using the small Seitz No. 6 filters (19 $cm^2$) employed routinely for laboratory purposes, and correlation of the results with the data obtained on a Seitz No. 200 filter press (up to 2240 $cm^2$) seemed warranted. The latter was used to process larger quantities of media which were stored until needed. The use of the larger filter measurably lessened the work load in media preparation and enabled enough media to be made at one time for control comparisons at desired intervals.

A series of filtrations were made on different batches of media at varying pressures. The filtration data were plotted by the methods proposed by Hermans and Bredée[7, 8] to determine the linear relationship most applicable to the filtration system. The cake law, which applies to most industrial filtrations, the standard law, the law of complete blocking and the intermediate law were tested by use of the filtration data.

The medium used for these tests was that mainly employed in the tissue culture studies. This consisted of 89 per cent Eagle's medium,[3] 10 per cent bovine serum and 1 per cent chick embryo slurry. The batches of media were tested immediately after preparation and also after a preliminary clarification which removed most of the particulate matter introduced by the chick embryo slurry. This approach was used to determine the effect of particulate matter on filter pad resistance and plugging rate, and to show the maximum flow rates which could be obtained in a final sterilization.

* Republic Seitz Filter Corporation.

A standard Seitz No. 6 laboratory vacuum filter (100 ml volume) was modified for use as a pressure filter by soldering a cover plate with a hose connection to the top of the filter. S–1T sterilizing pads, asbestos sheets manufactured and acid-washed by Republic Seitz Filter Corporation, were used as the filtering material. A pore size of 0·020 $\mu$ is listed by the manufacturer for S–1T pads. The surface area for this filter is 19·1 $cm^2$.

The freshly prepared tissue culture medium was fed through a peristaltic laboratory pump to the filter. A glass tee was inserted in the line between the pump and the filter to permit installation of a 0–15 $lb/in^2$ pressure gauge. Manual adjustment of the

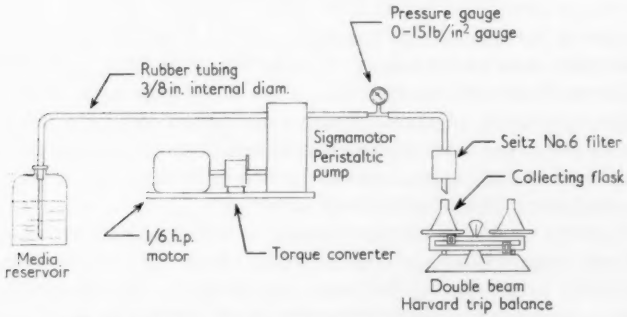


Fig. 1. Apparatus for measurement of filtration rates

torque converter gave adequate control over pressure variations so that a desired uniform pressure could be maintained. The filtrate was collected in a flask placed on a double beam trip balance. The experimental apparatus is shown in Fig. 1.

The filter was inverted while being filled with medium, to enable all entrapped air to escape. The desired pressure was reached and maintained by adjusting the pumping rate. The total filtration time, starting with the initial flow of filtrate, was recorded to the nearest second at each 20 g interval.

In addition to the experimental use of the small Seitz No. 6 filter, filtration rate data were obtained during the routine sterilization of stock media with the larger Seitz No. 200 filter press

containing S–1T pads. This filter press, when used for single filtration,* held up to seven asbestos sheets, each of which provided a filtering surface of 320 $cm^2$. The stock medium was collected in 400 ml volumes in sterile bottles. The pressure was maintained at the desired value while a 100 ml increment of filtrate was timed as each bottle was filled.

All media processed in the Seitz No. 200 filter press since the experiments on the Seitz No. 6 filter contained chick embryo slurry which previously had been filtered twice. This preliminary filtration ensured the absence of contamination with the deformable pleuropneumonia-like organisms (PPLO) which had been shown in earlier work to pass the S–1T pad when the organisms were present in large concentration. Bovine serum was always filtered twice through S–1T pads before the final sterilization, for the same reason. Therefore there was no significant difference between these media and the previously clarified media used in the tests with the Seitz No. 6 filter.

## Results

The data from preliminary filtrations were plotted, by the necessary methods, to test the applicability of each of the four filtration laws proposed by Hermans and Bredée.[7, 8] Plotting of the data by the method for the law of complete blocking ($S$ vs. $V$) resulted in irregular curves which were of no value in characterizing the filtration system. Similarly, the intermediate law plot ($1/S$ vs. $t$) was inapplicable. Plotting of the data by the standard law method ($t/V$ vs. $t$), however, resulted in a linear relationship from which constants characteristic of the filtration system were obtained. Filtration data from a typical batch of medium filtered at several pressures are plotted according to the standard law in Fig. 2. The same data resulted in smooth curves when plotted according to the cake law ($t/V$ vs. $V$), as shown in Fig. 3.

An attempt was made to relate the percentage of solids in each batch of medium to filtration behaviour, but the available method for determination of the percentage of solids was not sufficiently

* The Seitz No. 200 filter press can be made, with the use of bypass plates, to filter a fluid either once, twice, or three times, within the same operation if so desired.

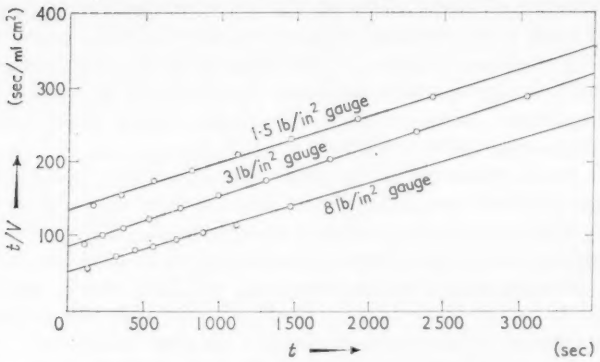


Fig. 2. Filter resistance vs. time in filtration of unclarified tissue culture media (standard law)

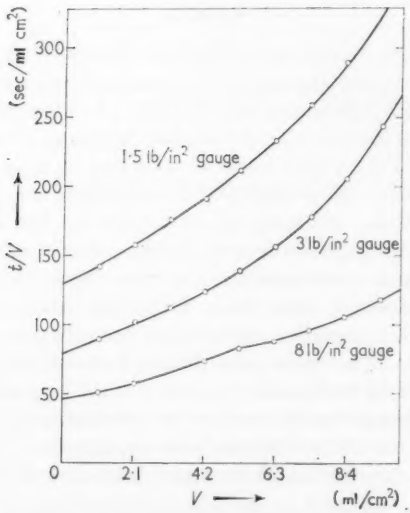


Fig. 3. Filter resistance vs. filtrate volume in filtration of unclarified media (cake law)

accurate for differentiation. The percentage of wet solids in the unclarified medium was within the range of 0·6–1·0 per cent, while the previously clarified medium contained only a slight trace of solids. The viscosities of several different batches of medium were measured with an Ostwald–Fenske Viscosimeter. There was no measurable difference in viscosities and the batches of medium were found to have a viscosity of 0·885 centipoise at the normal filtration temperature of 27°C.

In Table I are listed the average values of the standard law slopes and intercepts obtained from the curves plotted with the data from three different batches of medium which were filtered in both the unclarified and previously clarified states. The

Table I. Average values of standard law intercepts and slopes for three batches of medium

| $\Delta P$, lb/in² gauge | Unclarified media | | Previously clarified media | |
|---|---|---|---|---|
| | $k/2$ (slope), cm²/ml | $1/S_0$ (intercept), sec/ml cm² | $k/2$, cm²/ml | $1/S_0$ sec/ml cm² |
| 2·0 | 0·065 | 159 | 0·034 | 123 |
| 5·0 | 0·062 | 90 | 0·031 | 71 |
| 10·0 | 0·057 | 58 | 0·023 | 46 |

pressures were 2, 5 and 10 lb/in² gauge. The data from tests of one of these batches is plotted by the standard law method in Fig. 4 (unclarified) and in Fig. 5 (previously clarified). Individual values of slopes and intercepts were read from these graphs. The intercepts indicate the initial resistance to flow, and the slopes show the plugging rate. They were characteristic of the type of medium filtered.

The average values of the standard law intercepts listed in Table I are plotted as a logarithmic function of the differential pressure in Fig. 6. The average intercepts for the unclarified medium fell upon a perfectly straight line represented by the equation:

$$1/S_0 = 246\ \Delta P^{-0\cdot627}$$

in which $1/S_0$ is the standard law intercept, in sec/ml cm² and $P$ is the filtration pressure in lb/in² gauge. The average intercept value for the previously clarified medium at 2 lb/in² gauge fell

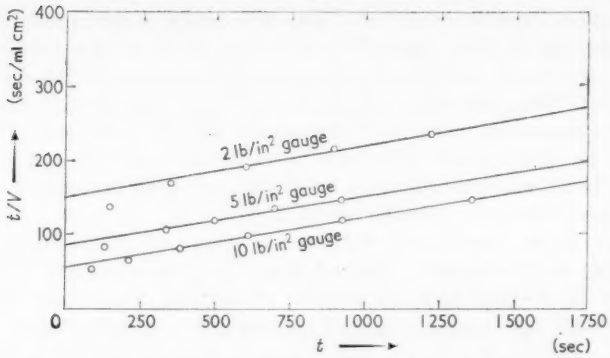


Fig. 4. Standard law plot for unclarified media (Batch No. 3)

slightly below a line passing through the points at 5 and 10 lb/in² gauge, and having exactly the same slope as the unclarified medium line. However, if one of the three intercepts determined at 2 lb/in² gauge were considered in error, the average value of the remaining two intercepts was found to fall on the line with the 5 and 10 lb/in² gauge.

The batches of previously clarified medium were thus represented by the equation:

$$1/S_0 = 195\,\Delta P^{-0.627}$$

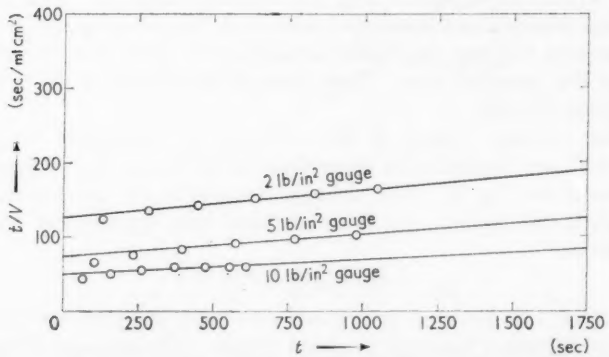


Fig. 5. Standard law plot for previously clarified media (Batch No. 3)

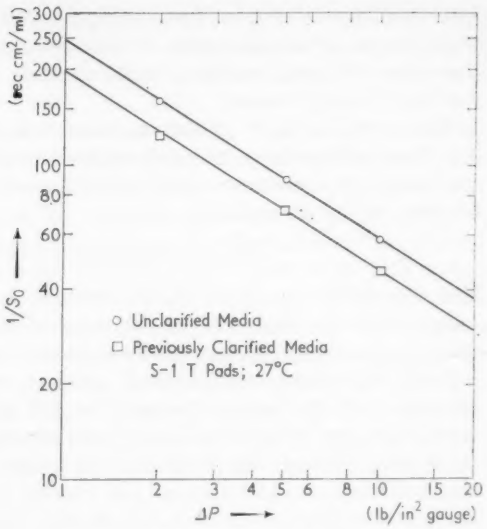


**Fig. 6. Standard law intercept, $1/S_0$, as a function of filtration pressure**

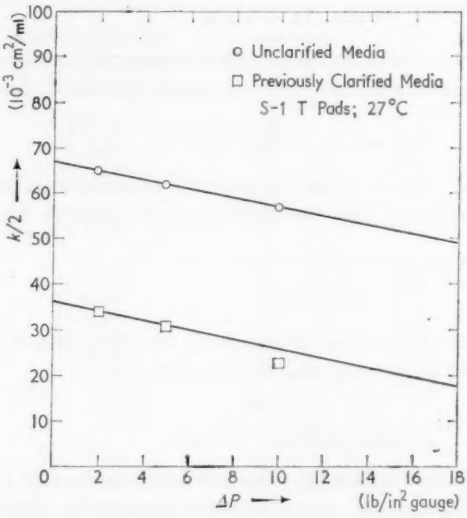


**Fig. 7. Plugging rate, $k/2$, as a function of filtration pressure**

The fact that the two lines were parallel indicates that the exponent of $\Delta P$ is a function of the filtering medium only, while the proportionality constant (195) depends on the properties of the liquid being filtered.

The average values of the standard law slopes are plotted in Fig. 7 as a function of the differential pressure. The data from the unclarified medium when plotted produced a straight line represented by the equation:

$$k/2 = -0{\cdot}001\ \Delta P + 0{\cdot}067$$

The standard law slope $k/2$ in cm²/ml, indicates the rate of plugging of the filter sheet. Because of the rapid flow of the filtrate at 10 lb/in² gauge, determination of an accurate value of $k/2$ for the previously clarified medium was difficult at this pressure. If the value obtained for $k/2$ at this pressure were assumed to be 10 per cent in error, a perfectly straight line could be drawn through the experimental points for the previously clarified medium, which again had exactly the same slope as the line representing the unclarified medium. The previously clarified medium were thus represented by:

$$k/2 = -0{\cdot}001\ \Delta P + 0{\cdot}036$$

The expressions of standard law slopes and intercepts in terms of pressure were substituted into the general standard law equation:

$$kt/2 = t/V - 1/S_0$$

where $V$ is the filtrate volume $V_f$, in ml, per unit filtering area $A$, in cm². Rearrangement of the standard law to express the time $t$, resulted in:

$$t = \frac{1/S_0}{A/V_f - k/2}$$

Substitution of the expressions determined for $1/S_0$ and $k/2$ in terms of $\Delta P$ for the previously clarified medium gave:

$$t = \frac{3{\cdot}25\ \Delta P^{-0{\cdot}627}}{A/V_f + 0{\cdot}001\ \Delta P - 0{\cdot}036}$$

in which $t$ is expressed in minutes. Similar equations may be written for the filtering area $A$, or the filtrate volume $V_f$. Thus

any one of the three conditions, $t$, $A$ or $V_f$ may be determined when the other two and $\Delta P$ are known. The expressions for $1/S_0$ and $k/2$ determined for the unclarified medium may be used in a similar manner.

Filtration data were recorded during routine sterilizations, through the Seitz No. 200 filter press, of medium for stock cultures. In Table II the periods of time required for sterilization of medium with the Seitz No. 200 filter press are compared with the times calculated from information obtained from the experimental filtrations through the Seitz No. 6 filter. The differential pressures listed have been corrected for gauge errors and back pressure. Some sterilizations involved batches of media containing chick embryo slurry which was previously clarified by filtration twice prior to its incorporation into the complete medium. The length of time required for each of these sterilizations was compared with the period calculated from results of experimental filtrations of previously clarified media through the Seitz No. 6 filter.

Table II. Comparison of measured filtration rates during sterilizations through the Seitz No. 200 filter press with rates calculated from Seitz No. 6 test results

| Sterilization date, month, day, year | $\Delta P$ (corrected), lb/in$^2$ gauge | $V_f/A$, ml/cm$^2$ | $t$, min calculated | $t$, min measured |
|---|---|---|---|---|
| | | Unclarified medium | | |
| 6.11.58 | 1·3 | 3·18 | 13·8 | 15·7 |
| 6.18.58 | 1·5 | 2·50 | 9·5 | 13·5 |
| 7. 3.58 | 1·3 | 2·25 | 9·0 | 11·3 |
| 8.14.58 | 1·9 | 2·92 | 9·8 | 12·0 |
| 8.29.58 | 2·5 | 4·59 | 15·0 | 20·0 |
| 9.11.58 | 2·5 | 4·17 | 13·1 | 17·5 |
| | | Previously clarified medium | | |
| 11.19.58 | 1·4 | 7·50 | 27·2 | 30·0 |
| 11.24.58 | 1·6 | 4·17 | 11·9 | 11·3 |
| 11.26.58 | 1·6 | 4·17 | 11·9 | 11·2 |
| 12. 9.58 | 1·0 | 3·90 | 14·8 | 13·0 |
| 1.21.59 | 1·2 | 4·60 | 16·0 | 16·7 |

## Discussion

Filtration of the unclarified medium conformed to the Hermans–Bredée standard law quite well. The previously clarified medium,

when refiltered, conformed to the cake law as well as it did to the standard law despite the fact that no cake was actually formed. This behaviour is not unusual when there is very little particulate matter in the feed and has been observed by Grace[5] and others.

As Grace noted in his filtration systems, the initial period of medium filtration through the Seitz S–1T sterilizing sheets did not conform to any theoretical filtration law. In all cases in which filtration was continuous, there was a sharp increase in effective pad resistance during the initial stage of filtration. On a $t/V$ vs. $t$ plot, this resulted in a smooth curve of decreasing slope which merged into the standard law region. A possible explanation for this initial behaviour may be found in the electrokinetic effect of streaming potential.

The influence of electrokinetic forces on filter performance has been discussed by Ruth.[12] The resistance of a filtering medium is usually greater during filtration of liquids than would be expected on the basis of calculation of pore length (cake law) or pore radius (standard law). The best explanation of this behaviour appears to be in that a streaming potential forms across the depth of the filtering medium and resists the flow of filtrate. This potential is set up by adsorption of $OH^-$ ions and negatively charged particles on the pore walls. Positive ions are then carried along by the stream and form an excess near the outlet end of the pores. This results in a potential difference which tends to reverse the flow of the positive ions and thus interferes with the free passage of the filtrate stream.[12, 14]

Abramson[1] calculated the probable error introduced by the electrokinetic effect in the viscometric measurement of a dilute salt solution through glass capillaries. He found that for capillaries $1 \times 10^{-4}$ cm in radius, the error is as much as 10 per cent. The error due to retardation by the streaming potential increases rapidly with smaller radii.

It is probable that the failure of the initial period of filtration to conform to either the standard law or the cake law is due to the streaming potential. The pore sizes in S–1T sterilizing sheets are very small (20 m$\mu$) and the effect of streaming potential becomes much greater for smaller capillary radii. A period of flow through a capillary is necessary for the build-up of a streaming potential. For this reason, the streaming potential builds up from zero at the

start of filtration to a maximum value during filtration. The sum of the effects of mechanical plugging and increasing streaming potential cause a rapid increase in effective pad resistance during the initial stage of filtration. As the streaming potential approaches its maximum value, however, the rate of increase of resistance is due only to plugging and can be determined from a standard law plot of the filtration data.

The streaming potential is a function of the pressure drop across the filter pad. This increase in streaming potential acts along with compression of the filtering medium to decrease the effect of increased pressure on flow rate. Thus, doubling the filtration pressure will not double the flow rate, which in the system investigated varied as $\Delta P^{0 \cdot 627}$.

In a review of ultrafiltration, which included a discussion of the mechanism of ultrafiltration of disperse systems, Ferry[4] noted that increase of pressure narrows the zone of primary adsorption. This takes place presumably by defining more closely against the filter support the areas through which flow can take place and by a tendency for the pressure to shear away adsorbed layers in the pores.[4] The observed decrease in plugging rate with increasing pressure may thus be explained. The force necessary to shear adsorbed particles from pore walls develops nearer the walls at greater pressures. Thus the plugging effect of adsorbed particles is lessened.

Ferry also noted that the effect of blocking is reduced by lowering the pressure and apparently disappears at zero pressure (dialysis). The pores of asbestos filter sheets are not of uniform diameter throughout and contain restrictions at which blocking may occur and affect the plugging rate. Decreased blocking effects might account for an apparent decrease in plugging rate as the filtration pressure is dropped below 2·0 lb/in$^2$ gauge. A plot of standard law slopes for runs in both the Seitz No. 6 and No. 200 filters indicated a maximum plugging rate at 2·0–3·0 lb/in$^2$ gauge. Thus, the equation relating $k/2$ to $\Delta P$ may be in error at pressures below 2·0 lb/in$^2$ gauge.

The equations for filtration time $t$ in terms of $\Delta P$, $A$ and $V_f$, which were obtained from tests with the small Seitz No. 6 filter, were used to calculate the theoretical times for routine medium sterilizations in the Seitz No. 200 filter press. The times

calculated for the unclarified medium filtrations were consistently less than the measured values but the correlation was sufficiently accurate for engineering requirements. The accuracy of the calculated times for the previously clarified medium was very good, being slightly better than that obtained by McMillen and Webber[10] in correlating two filters of different sizes in a cake-type filtration system. All recent sterilizations have been of medium of the previously clarified type and the methods of filter press operation and data collection have been improved materially since the sterilizations involving the unclarified medium.

Should the need arise for a much larger supply of sterile tissue culture medium than is now being used, the results of this investigation would prove invaluable for calculating data. The filter size required to sterilize a specified volume in a specified length of time can be calculated readily for any filtration pressure. In addition, since the variation of standard law slopes and intercepts with filtration pressure was found to be the same for different types of media in the Seitz S–1T system, the results of a single small-scale test of another type of medium could be used for calculations at any filtration pressure.

## Conclusion

A tissue culture medium containing Eagle's medium, bovine serum and chick embryo slurry was tested by means of a series of constant pressure filtrations in a small Seitz No. 6 laboratory filter containing S–1T asbestos pads. Preliminary tests showed that, of available filtration theory, the Hermans–Bredée standard law postulating a gradually increasing blocking of the pores best described the rate data. The constants for the standard law equation were determined for a medium containing unfiltered chick embryo slurry and for a clarified medium. Three different batches were tested at filtration pressures of 2, 5 and 10 lb/in$^2$ gauge and the data were plotted by the standard law method of analysis. The average values of slopes and intercepts were related to filtration pressure by graphical means.

The intercepts, or effective initial filter resistances, were expressed by the relation $1/S_0 = k\,\Delta P^x$. Characteristic values of

the constant $k$ were determined for the clarified and unclarified media; the value of $x$ was found to be $-0{\cdot}627$ in both cases. Similarly, for the slopes or plugging rates, $k/2 = C'\Delta P + C''$ was characteristic of the type of medium filtered; $C'$ was equal to $-0{\cdot}001$ in both cases. Thus one of the constants in each equation is characteristic of the filtering medium only.

The expressions for $k/2$ and $1/S_0$ in terms of $\Delta P$ were substituted into the basic standard law equation. From the resulting equation, any one of the three conditions of filtration time, volume of filtrate, or filtering area could be calculated when the other two conditions and the filtration pressures were specified. Much filtration data has been obtained during the sterilization of media for stock culture in a Seitz No. 200 filter press. The recorded filtration times were compared with times calculated from the experimentally determined equations. Agreement between measured and calculated values was very good for the previously clarified medium and within engineering requirements for the unclarified medium.

The initial part of each filtration did not conform to any of the basic filtration laws. A possible explanation for this may be found in the electrokinetic effect of streaming potential. The variations of the initial filter resistances and plugging rates with the filtration pressure were also discussed on the basis of theoretical filtration mechanisms.

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## *Note*

# Disruption of Frozen Bacteria as a Consequence of Changes in the Crystal Structure of Ice*

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*Summary.* It is shown that at $-25°C$ alterations in the crystal structure of ice caused by repeated pressure changes in the regions of 2000 and 3500 kg/cm$^2$ are accompanied by a disruption of *E. coli* cells suspended in the ice.

To prepare biological material for extraction it is common practice to subject it to a sequence of freezings and thawings (*cf.* Billaudelle *et al.*[1]). In the latter process the material is frequently exposed to relatively high temperatures in order to save time, and this may destroy important constituents of the preparation. When considering how to achieve cell disruption at a lower temperature than around $\pm 0°C$ we decided that not only the effect of the phase-shift liquid/solid but also of possible solid/solid transitions had to be investigated.

It is a well known physico-chemical phenomenon that, at certain temperatures, when ice is subjected to high pressure, it changes from being lighter than water to a state where it is much heavier.[2,3,5] The careful examination of the relation between temperature, pressure, and volume has permitted the definition of many different forms of ice (Fig. 1.) At $-22°C$ and 2115 kg/cm$^2$, ice I (ordinary ice) is transformed into ice III, the change causing a decrease in volume of 0·182 cm$^3$/g. It seemed likely that this transformation of the crystal-structure might be able to disintegrate micro-organisms suspended in the ice.

* The research reported in this paper was supported by a grant from the Wallenberg Foundation.

## Methods

*Escherichia coli B* was chosen as test-organism. It was grown in a synthetic medium[4] at 37°C and harvested at the end of the logarithmic growth phase ($5{\cdot}6 \times 10^9$ bact./ml). The culture was cooled, centrifuged and washed twice in a refrigerated centrifuge (3,000*g*). The sediment was then frozen down to −25°C and stored at this temperature for about a fortnight. It was then suspended in a buffer (436 ml, 0·2 M $Na_2HPO_4$ + 65 ml, 0·1 M citric acid + 500 ml, 0·1 M sodium citrate + 1000 ml Aq.dest., pH = 7·4) to a concentration of $10^{11}$ bact./ml. The suspension

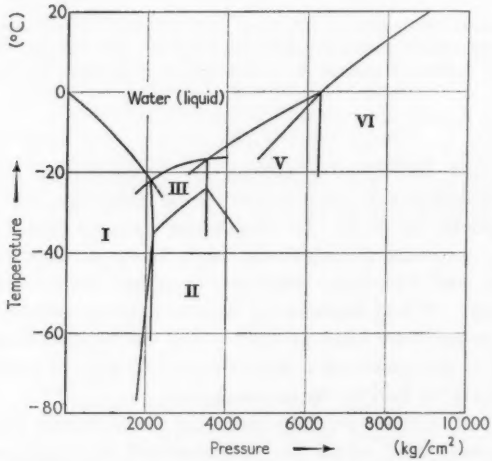


Fig. 1. Different forms of ice. (From P. W. Bridgman. Water in the liquid and five solid forms, under pressure. *Proc. Amer. Acad. Arts Sci.*, **47**, 439 (1912))

was distributed in Petri dishes containing 25 ml each, immediately frozen and then kept at −25°C until used. For each experiment one Petri dish was taken, the contents just thawed, 10 ml used for the pressure experiment, and the rest frozen again to serve as a control.

The pressure chamber consisted of a hole, 85 mm deep and 20·4 mm in diameter, which was drilled into the round end (diam. 150 mm) of a cylindrical steel block weighing just above 20 kg. A

steel plunger (Fig. 2) fitted perfectly in the hole. It was fitted with a teflon gasket sandwiched between a soft iron washer and the separate chamber end of the plunger. The latter had a neck surrounded by the washer and gasket, which fitted into an axial hole in the cylindrical main body of the plunger. This hole was deeper than the length of the neck. The neck and, in fact, the whole chamber end of the plunger, were also perforated by an

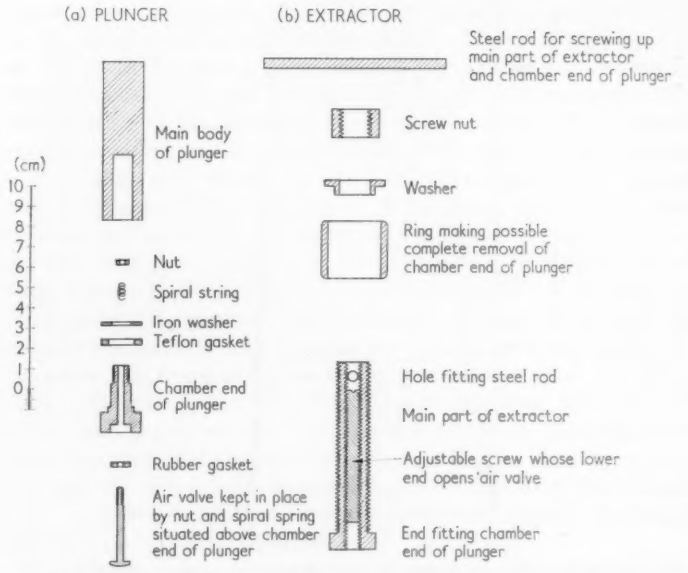


Fig. 2. (a) Complete plunger, (b) tool for extraction of chamber end of plunger ('Extractor')

axial hole through which the stem of the tack-like air valve protruded.

In the pressure-chamber the air over the suspension was released and the chamber cooled to $-25\,^{\circ}C$. Pressure was then applied with the aid of a hydraulic jack. In the region of the phase boundaries there was no corresponding alteration in the

pressure read on the jack manometer when the volume was altered, until all ice had changed phase. The boundaries obviously were somewhat higher when the pressure was increased, than when it was decreased. Those empirically found boundaries were used when the experiments were carried out, *i.e.*, experiments 2 and 4 were performed by repeatedly crossing the boundaries between phases I–III and III–V, respectively, while in the other

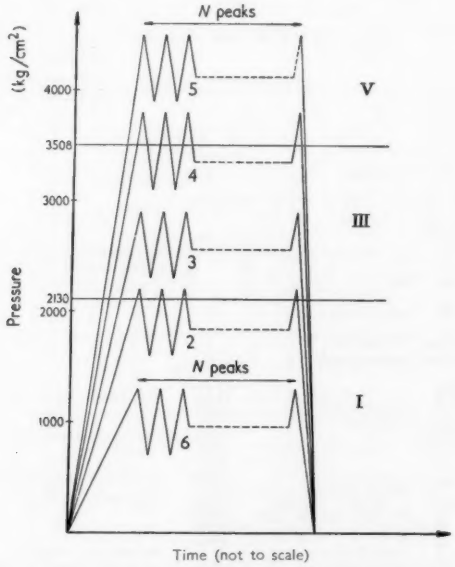


Fig. 3. Pressure applied in the different experiments (exps. 2–6)

experiments (3, 5, 6) those boundaries were avoided (see Fig. 3).

After the pressure treatment both the pressure-exposed and the control suspension which had been frozen at the same time at atmospheric pressure, were thawed, diluted 1/100 with the phosphate-citrate buffer and kept in an ice-bath for two hours in order to allow time for the soluble part of the material and its sedimentable portion to reach an equilibrium. The samples were then spun down into a refrigerated centrifuge at 25,000*g* for half

an hour. The nitrogen content of the undiluted supernatant was determined by micro-Kjeldahl while the extinction values at 260 and 280 mμ were obtained in a Beckman DU Spectrophotometer on the samples diluted 1/100. The values are listed in Table I. Specimens for electron microscopy were prepared from the sediments.

*Results and discussion*

From Table I it can be seen that when the phase boundaries are passed repeatedly (experiments 2 and 4) the bacteria are subjected to the most pronounced disintegration. This is especially true for the phase boundary between ice I and III (exp. 2) where the volume variation is also greatest: 0·185 $cm^3/g$, as compared with 0·0546 $cm^3/g$ at the boundary between III and V. The values of experiments 1, 3 and 5 where the phase boundary has been passed only once in each direction, are lower. However, it is obscure why the values from experiment 5 are lower than those from experiment 1 although the suspension is treated in the same way in both, with the exception of nine additional pressure variations being imposed on the suspension in experiment 5. It might be due to some experimental error perhaps involving the speed of the pressure release. When the pressure was suddenly released a jarring noise was frequently heard when passing the phase boundary I–III. In the experiments this phenomenon was, however, not taken into consideration. In experiment 6 no disintegration exceeding that of the control was expected as no phase boundary was passed. The slight compressibility of ordinary ice without phase variation might be responsible for the slightly increased disintegration in this experiment.

The disintegration obtained in experiment 7 at 12°C is very small as compared to the effect of a similar series of pressure changes at −25°C. The difference is easily understood if one remembers that at −12°C the phase shift I–III cannot occur. The small effect at −12°C is easily explained by the change from ice I to water, which is followed by a volume decrease of almost 0·12 $cm^3/g$.

When the bacterial suspension in ice is compressed, friction of course occurs between the mantle surface of the ice and the steel hole. A disintegration effect as a consequence of this friction was

Table I. Experimental conditions and results

| Experiment | Treatment | | | | | | Extract | | |
|---|---|---|---|---|---|---|---|---|---|
| | Temp. °C | Pressure changes ($n$) | Pressure (kg/cm$^2$) Max. | Min. | Phase boundary passages I–III | III–V | Extinction* 260 mμ | 280 mμ | mg N/ml |
| 1 | −25·5 | 1 | 3900 | 0 | 1 | 1 | 0·361 | 0·175 | 0·76 |
| 2 | −25 | 10 | 2200 | 1600 | 10 | 0 | 0·528 | 0·265 | 0·96 |
| 3 | −25 | 10 | 2900 | 2300 | 1 | 0 | 0·345 | 0·170 | 0·82 |
| 4 | −25 | 10 | 3800 | 3100 | 1 | 10 | 0·364 | 0·182 | 0·86 |
| 5 | −25 | 10 | 4500 | 3900 | 1 | 1 | 0·321 | 0·155 | 0·67 |
| 6 | −24·5 | 10 | 1300 | 700 | 0 | 0 | 0·195 | 0·086 | 0·33 |
| 7 | −12 | 10 | 3900 | 0 | 0 | 0 | 0·309 | 0·147 | 0·57 |
| Untreated bacteria | −25 | — | — | — | — | — | — | — | 2·76 |
| Supernat. from untreated bacteria | −25 | — | — | — | — | — | 0·140 | 0·061 | 0·19 |

The number of pressure changes ($n$) are the same as those indicated in Fig. 2. The number ($n$) indicates the number of complete (rise + fall) changes. The maximal and mininal pressures in the following column indicate the borders of the pressure change sequence (Fig. 2). In the sixth and seventh column the number of phase boundary passages in each direction are listed both for ice I–III and for III–V.

* Measured in a 1/100 dilution of extract, after centrifuging.

ruled out by rinsing off the surface layer of the ice after the pressure treatment and before thawed material was taken for analysis.

The phase diagram of ice (Fig. 1) shows that under certain conditions the border lines between, for instance, water and ice I, and water and ice III, are extended into ice III and ice I, respectively.

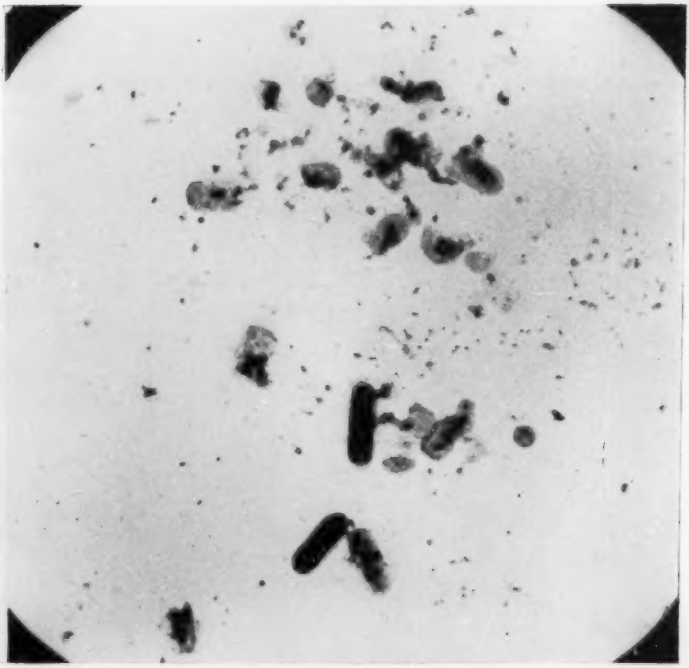

Fig. 4. Electron micrograph showing bacteria treated by repeated phase boundary passages. Cell walls as well as undisrupted cells are seen. (Magnification 6,000 times)

However, the transition of ice generally proceeds very fast near the triple point (Water–Ice I–III). Whether the presence of bacteria or their products might influence the equilibria between those forms of ice cannot be stated at the present time.

An electron micrograph (Fig. 4) indicates that the cell-wall

damage caused by phase shift was not very conspicuous in spite of the fact that the bacteria seem to be quite empty.

Evidently a large number of bacteria subjected to the phase variations of ice at $-25°C$ are disintegrated. Whether this method might be applicable to practical disintegration purposes must be investigated further. The effect on small particulate systems (e.g. phages and viruses) will also be studied.

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## Errata

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p. 328 For '(c) moisture content of the filter ($w$ = 0–84 per cent)' read '. . . ($w$ = 0–83 per cent)'

p. 331 Plotted line should be turned clockwise through 180°, relative to the coordinates

pp. 332 and 333 Figs. 4 and 5 only should be *interchanged*, captions as stand